APPLICATION OF BACTERIOPHAGE-DISPLAYED PEPTIDE LIBRARIES TO STUDY POLYSACCHARIDE ANTIGENS OF *AEROMONAS SALMONICIDA*

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DECLARATION

This thesis is the original work of the author:

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DEDICATION

This thesis is dedicated to my family, friends and my wife Katherine for their support and encouragement throughout my undergraduate and post graduate years!.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABTS	= 2,2'-Azino-di-(3-Ethyl-Benzthiazoline sulphinate)
BAC-sulfoNHS	= biotinamidocaproate-N-hydroxy-sulfosuccinimide ester
BSA	= bovine serum albumin
CFU	= colony forming units
CPS	= capsular polysaccharide
dH_2O	= distilled water
ELISA	= enzyme linked immunosorbent assay
HABA	= hydroxyazobenzene-2-carboxylic acid
HRP	= horse radish peroxidase
Km	= kanamycin
Km^r	= kanamycin resistant
LB	= Luria broth
LPS	= lipopolysaccharide
MAb	= monoclonal antibody
ORF	= open reading frame
PB	= phosphate buffer
PBS	= phosphate buffered saline
PCR	= polymerase chain reaction
PEG	= polyethylene glycol
r.p.m	= revolutions per minute
TBS	= tris buffered saline
Tc	= tetracycline
Tc^r	= tetracycline resistant
TU	= transducing units
wt	= wild type

SUMMARY

This thesis describes the application of bacteriophage displayed peptide libraries to the salmonid fish pathogen *Aeromonas salmonicida*. Antisera to both the capsular polysaccharide and lipopolysaccharide of *A. salmonicida* were used to select phage from 6-mer and 15-mer bacteriophage peptide libraries displayed in the p III protein of bacteriophage fd tet. When rabbit polyclonal anti-capsular polysaccharide antiserum was used for selection of phage from the 6-mer library, 140 phage were recovered by elution with glycine /HCl buffer (first round elution) and capsular polysaccharide solution in the second and third round of elution. Phage were amplified to high concentration and analysed by enzyme linked immunosorbent assay (ELISA) and the insert sequences determined

Biopanning with anti-capsular polysaccharide antiserum selected a range of phage with peptide insert sequences which contained similar 3 to 4 amino acid motifs. The most predominant motif was 'serine-glycine-serine'. ELISA demonstrated that several of these phage bound to anti-capsular polysaccharide antiserum suggesting that these phage peptide sequences might antigenically mimic some capsular polysaccharide epitopes to which antibodies were present in the antiserum.

A monoclonal antibody to *A. salmonicida* lipopolysaccharide was also used for biopanning with the 6-mer library and this selected phage with sequences which could be classified into groups, several containing identical sequences and others with similar motifs. ELISA assays to detect binding of these phage to lipopolysaccharide antiserum were inconclusive. Several selected with anti-lipopolysaccharide monoclonal antibody displayed peptides similar to those selected by polyclonal anti-capsular polysaccharide antiserum, which may reflect the similar monosaccharide composition of these related polysaccharides.

1. INTRODUCTION

Commercial salmon farming in Scotland began over 30 years ago and the industry has developed from producing 300 tonnes of fish in 1980 to over 86000 tonnes in 1997 (G. Rae, Scottish Salmon Growers Association, personal communication). The value of this product is approximately £250 million per annum and the industry now employs over 2000 people. Among the constraints to growth of the industry has been the impact of infectious diseases (Hastein, 1995), and the increasing population of farmed salmon serves as a ready target for infectious agents. Amongst the best known infectious agents are *Aeromonas salmonicida* (causing furunculosis), *Vibrio anguillarum* (causing vibriosis), *Yersinia ruckeri* (enteric red mouth disease), and infectious pancreatic necrosis virus (IPN, a birnavirus) (Hastein, 1995). In addition to microbial infections, infestation by sea lice is currently one of the greatest problems causing weight loss and mortalities.

1.1 Microbial Diseases in Salmon aquaculture

There are many reviews on the impact of diseases on aquaculture and Table 1 is adapted from Hastein (1996). It should be noted that ISA virus has recently been isolated in Scotland.

1.2 Furunculosis

The first documented evidence of furunculosis was over 100 years ago by Emmerich and Weibel 1894 (cited by Bernoth, 1997). In the winter of 1888/89, furuncle-like swellings that led to ulcerative lesions were noted in brown trout in a German fish farm. Bacteria were subsequently isolated from the ulcerated lesions and a purified culture of the organism was shown to induce the same pathology as the natural infection when introduced into uninfected fish. The trout 'epizooty' was known to resemble *Vibrio cholerae*, and was initially named *Bacterium salmonicida* (Emmerich & Weibel, 1894). The causative organism was subsequently renamed *Aeromonas salmonicida* by Griffin *et al.* (1953; cited by Bernoth, 1997). Emmerich & Weibel described the organism as a rod-shaped, gram-negative, non-motile, facultatively

Pathogen	Host	Disease	Environment
Viruses			
IHN virus	Pacific salmon,	Infectious Hematopoietic Necrosis (IHN)	North America, Japan, Europe
VHS virus	Rainbow trout	Viral Haemorrhagic Septicaemia (VHS	Europe, USA
ISA virus	Atlantic salmon	Infectious Salmon Anaemia (ISA)	Norway
Bacteria			
Yersinia ruckeri	Salmonids	Yersiniosis (red mouth)	North America, Europe
Vibrio anguillarum	All fish	Vibriosis	Ubiquitous
Vibrio salmonicida	Atlantic salmon	Cold water vibriosis	Europe, North America
Aeromonas salmonicida	Salmonids	Furunculosis	Ubiquitous, fresh & sea water
Renibacterium salmoninarum	Salmonids	Bacterial kidney disease (BKD)	North America, Europe, fresh & sea water.
Mycobacterium	All species	Fish 'tuberculosis'	Ubiquitous, fresh & sea water
Piscirickettsia salmonis	Pacific and Atlantic salmon	Salmonid rickeutsial septicemia (SRS)	North and South America
Fungi			
Exophiala spp.	Salmonids	Visceral mycosis	Ubiquitous
Ichthyophonus hoferi	All fish	Visceral mycosis	Marine environment
Parasites/Protozoans			
Ichthyobodo necator	All fish	Skin, gill infection	World wide, fresh & seawater
Myxobolus cerebralis	Salmonids	Whirling disease	Europe, USA, fresh water
PKX	Salmonids	Proliferative kidney disease (PKD)	Europe, North America
Gyrodactylus spp.	All fish	Skin, gills	World wide, fresh water
Eubothrium spp.	Salmonids	Gut	Fresh water, sea water
Lepeophtheirus spp.	Marine fish	Skin	Sea water
Caligus spp.	Marine fish	Skin	Sea water

Table 1. Diseases of economic importance in salmonids Adapted from Hastein (1996).

anaerobic bacterium which was unable to grow at 37°C. They also observed that after a few days growth on gelatin media the organism produced a diffusible brown pigment. This was verified by Marsh (1902) who noted that the organism was pleomorphic with dimensions within the ranges 0.5-6.0 x 0.5-1.0μm.

1.2.1 Taxonomy of *Aeromonas salmonicida*

Currently the species *A. salmonicida* is divided into subspecies: subsp. *salmonicida*, subsp. *achromogenes*, subsp. *masoucida* and subsp. *smithia* (Popoff, 1984; Austin *et al.*, 1989). The 'typical' strains which causes furunculosis are those of *A. salmonicida* subsp. *salmonicida* and the rest are considered as 'atypical' subspecies. The atypical strains can however still cause ulcerations, as demonstrated by the (as yet) unclassifed 'atypical' cytochrome oxidase-negative *A. salmonicida* isolated from ulcerated flounders (Wiklund *et al.*, 1994). It has been suggested that this organism should be located in a new subspecies. Typical and atypical strains of *A. salmonicida* have been compared using biochemical reactions, phage sensitivity and serological relatedness (Paterson *et al.*, 1980), however, *A. salmonicida*. subsp. *salmonicida* is the organism mainly discussed in this review.

1.2.2 Pathology of the disease

Furunculosis in salmon usually occurs as an acute bacteraemia, which makes the fish's organs prone to colonisation by *A. salmonicida* with resulting lesion formation. Visible lesions occur when the organism colonises the capillaries of the muscle or skin (Munro, 1988). There are various manifestations of the disease such as peracute, acute and chronic infections. In brief, a peracute infection is sudden and leaves little evidence of the disease; an acute infection is similar to the peracute infection but the animal shows increased symptoms, with the possibility of furuncle formation; finally, a chronic infection, which is more commonly found in older fish, is a prolonged infection with all the known symptoms of the disease including furuncles, leading to large cavities in the musculature of the fish (Munro, 1988). Fish which survive an outbreak of furunculosis may become asymptomatic carriers of the disease, whilst being apparently healthy (McCarthy, 1980). Such fish are prone to develop the disease

under natural stress, such as during smoltification and migration to sea. Factors such as crowding, transport to other sites, elevated temperature and poor water quality can also precipitate overt furunculosis. Scallon and Smith (1985) concluded that up to 100% of some populations of Atlantic salmon smolts were latent carriers of *A. salmonicida*. The location of the organisms in fish harbouring *A. salmonicida* in the latent state is uncertain, but recent work by Hiney *et al.* (1994) using bacteriological tests and enzyme-linked immunosorbent assays (ELISA) suggests that the organism is carried in the intestine and may also colonise the mucus, fins and gills .

1.2.3 Control of furunculosis

Various strategies have been adopted to reduce the impact of furunculosis (Bernoth *et al.*, 1997), and these include improved fish husbandry, genetic selection for disease resistance in fish, antibiotic treatments and vaccine development.

1.2.4 Fish husbandry

The most immediate way to prevent furunculosis outbreaks in a farm is by improving hygiene to prevent inadvertent importation of the bacterium into the farm environment. This involves complete sterilisation of equipment (boots, boats, etc.) before returning from another fish farm, and routine disinfection of fish ova which have come from outwith the farm, as trout ova were found to carry the organism on their outer surface but not internally (Mackie, 1930, quoted by Gee and Sarles, 1942).

Another important aspect of fish husbandry is the influence of diet on the potential immune response of fish. An example is provided by Teskeredzic *et al.* (1989) of high mortalities of fish in Yugoslavia which could be related directly to deficiencies of vitamins C and B2 in the fish diet. The relationship between diet and the immune response of fish is further reviewed by Landolt (1989).

1.2.5 Antibiotic treatment

Treatment of fish disease has for the most part relied upon the use of antibiotics, and at the height of the furunculosis epidemic in 1989 over 40 tonnes of antibiotics were used in Norwegian aquaculture, more than was used by humans in Norway (Lunestad *et al.*, 1992).

Such widespread use of antibiotics has inevitably led to the isolation of antibiotic-resistant strains of *A. salmonicida* and other pathogens. For example, Brazil *et al.* (1986) isolated strains of *A. salmonicida* which carried an Inc U plasmid coding for resistance to sulphadiazine, spectinomycin, streptomycin, trimethoprim and in some cases tetracycline. Antibiotic treatment of fish is normally done by incorporation of the agent in the diet, and studies by Samuelsen (1989) showed that only 20-30 % of added oxytetracycline was taken up by the fish, the majority finding its way into sediments below the cages, where it may persist for at least 3-6 months after treatment is stopped (Jacobsen and Berglind, 1988).

A subsequent problem is the reported transfer of the naturally occurring tetracycline resistance plasmid pRAS1 to *A. salmonicida* in marine sediments (Sandaa and Enger, 1994). Transfer of the plasmid occurs at higher frequency in the presence of the selective factor, oxytetracycline, leading to the conclusion that over-usage of antibiotics is increasing the occurrence of resistant bacteria. This has led to increased interest in other methods of prevention and treatment of disease, such as selective breeding of salmon with genetic traits for increased immunity, and perhaps greater resistance to bacterial infection (Lund *et al.*, 1995; Marsden *et al.*, 1996).

1.2.6 Vaccine development

With the increased occurrence of antibiotic resistance, the availability of effective furunculosis vaccines is essential. The initial success of Duff (1942) in testing an oral vaccine against *A. salmonicida* led to the hope that an effective vaccine would be produced commercially. Early developments have been reviewed by Hastings (1988), but only in the last decade have effective vaccines been developed (Ellis, 1997) and these are based on incorporation of oil-based adjuvants (Midtlyng, 1996, 1997), despite the significant side effects, such as reduced weight gain (Lillehaug *et al.*, 1992) and visceral adhesions (Midtlyng, 1996). Nevertheless, the effectiveness of vaccination programmes is shown by the statistic that in the Highland region of Scotland, smolt survival improved from 49% of those transferred to sea in 1991 to 96% for those transferred in 1993 (Munro and Gauld, 1996).

Currently-used vaccines appear to contain killed bacteria and inactivated extracellular products (Ellis, 1997) and the role of iron-regulated outer membrane proteins as key components has been emphasised by Bricknell and Ellis (1993). A different approach was adopted by Vaughan *et al.* (1993) who constructed mutants of *A. salmonicida* which were unable to synthesise aromatic amino acids (aro mutants) and could be used as live vaccines. The vaccine induced an immune response in salmon with no detectable signs of disease (Vaughan *et al.*, 1993; Marsden *et al.*, 1996), however, this vaccine has not been applied commercially.

1.3 Virulence factors of *Aeromonas salmonicida*

A. salmonicida produces an array of potential virulence factors, some of which are cell-associated and some which are extracellular products (ECP). Cell-associated factors include lipopolysaccharide, capsular polysaccharide, a unique additional protein layer (A layer), and outer membrane proteins; the ECP include at least 25 proteins, of which the serine protease and glycerophospholipid-cholesterol acyltransferase (GCAT) are the most important in terms of producing the pathology typical of furunculosis (Ellis, 1991). Paradoxically, it has recently been shown that mutants of *A. salmonicida* unable to produce active GCAT are still virulent for Atlantic salmon, as are mutants unable to produce the serine protease (McIntyre *et al.*, 1998). The ECP will not be discussed in detail but a detailed recent review of *A. salmonicida* ECP has been provided by Ellis (1997).

1.3.1 A layer

The A layer is composed of a regular tetragonal surface array composed of subunits of a 49 kDa protein (Trust, 1986, 1993), and its importance in the infection is shown by the loss of virulence of mutants lacking A layer (Ishiguro *et al.*, 1981). The A protein is very hydrophobic (Trust *et al.*, 1983), causing autoagglutination of the organism (Ishiguro *et al.*, 1981; Evenberg and Lugtenberg, 1982; Olivier, 1990), and is anchored to the cell suface by interaction with the LPS (Belland and Trust, 1985).

1.3.2 Lipopolysaccharide

Another major antigen of the cell envelope is the lipopolysaccharide (LPS) which is comprised of three major units: lipid A, a core oligosaccharide, and the O-polysaccharide, or O-antigen. The O-antigen chains are homogeneous in length and are antigenically monotypic (Chart *et al.*, 1984). In conjunction with the A-protein, LPS appears to neutralise the normal serum bactericidal mechanisms of fish towards *A. salmonicida* (Munn *et al.*, 1982). It has also been shown that LPS associates with the major lethal exotoxin, GCAT to stabilise it and enhance its activity (Lee and Ellis, 1990). Using techniques such as methylation analysis, periodate oxidation and proton magnetic resonance analysis, the structure of the O-antigen of LPS was deduced by Shaw *et al.* (1983), as shown in Figure. 1.

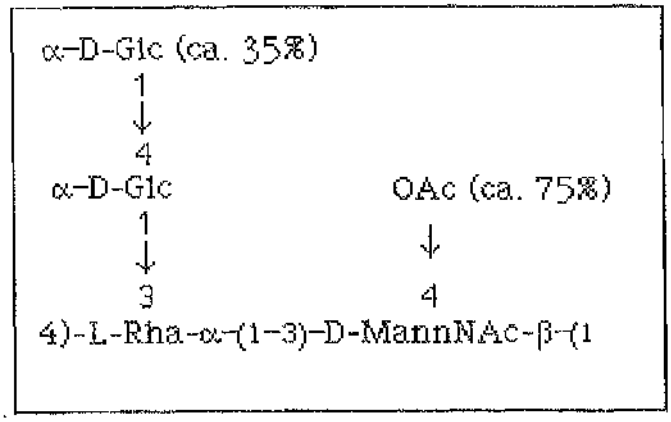


Figure 1. The structure of the repeat unit of Lipopolysaccharide. (after Shaw *et al.*, 1983)

1.3.3 Capsular polysaccharide

Under certain growth conditions *in vitro A. salmonicida* produces a slime layer or capsule (Garrote *et al.*, 1992) and this may also be expressed *in vivo* (Garduno *et al.*, 1993). It has also been shown that the possession of a capsule enhances adherence of the organism to, and invasion of, fish cell lines (Merino *et al.*, 1996), as well as enhancing resistance of *A. salmonicida* to killing by non-immune serum, thus increasing the survival of the organism in the blood and promoting septicaemic furunculosis (Merino *et al.*, 1997; Garduno and Kay, 1995).

Bricknell *et al.* (1997) showed recently that *A. salmonicida* possesses a surface polysaccharide which is antigenically and chemically distinct from the LPS and that can induce an immune response in fish. Similarities between this surface polysaccharide and the above-mentioned capsule suggest that they are the same component.

1.3.4 Outer membrane proteins

Proteins of the outer membrane of *A. salmonicida* were characterised by Evenberg *et al.* (1982), and proteins involved in iron uptake were described by Chart and Trust (1983), Aoki and Holland (1985) and Hirst and Ellis (1994). The iron-regulated outer membrane proteins are considered key constituents of vaccines protecting against furunculosis (Hirst and Ellis, 1994; Bricknell *et al.*, 1996).

1.4 Use of bacteriophage-displayed peptide libraries

The purpose of this thesis was to evaluate whether phage-displayed peptide libraries could be used to discover peptides which might mimic epitopes present on polysaccharide antigens. The models chosen for this work were the lipopolysaccharide (LPS) and capsular polysaccharide (CPS) of *A. salmonicida*. Although such antigens can be produced readily in culture they are convenient models for other polysaccharide antigens which could not be produced in the quantities required for vaccines.

1.4.1 Principle of phage-display peptide technology

The filamentous bacteriophage belong to the genus of non-lytic single-stranded DNA phages of the Inoviridae, also known as Inoviruses (definition from Singleton and Sainsbury, 1987) and their measurements range between 760 and 1950 nm in length and 6 to 7 nm in diameter. The bacteriophage fd is a member of the filamentous ff phage which include f1 and M13. The latter two differ in genome sequence from fd bacteriophage by only a few nucleotides.

Phage display of foreign peptides is possible because of the architecture of filamentous bacteriophage, in that the coat proteins encoded by gene VIII and gene III have surface exposed N-terminal domains that tolerate foreign peptide inserts (Smith, 1993). Thus, insertion of foreign peptides into coat proteins at a suitable location, allows the inserts to be expressed on the outer surface of the bacteriophage. This led to the development

of 'bacteriophage-displayed peptide libraries' where individual phage contains different insert sequences.

Bacteriophage fd is well suited to formation of a phage-displayed peptide library which can be screened to select phage which bind to a target molecule, such as a specific antibody, by the process of biopanning. Biopanning is the name given to the selection process in which phage that have an affinity for the bound target molecule are selected, recovered, amplified to high titre and further rounds of biopanning carried out to select bacteriophage which bind the target molecule with high affinity (Scott and Smith, 1990). Selected phage can then be sequenced to identify the peptide displayed on the surface of the phage. The sequences determined from isolated phage are of interest since they can reveal the specificity of antibodies and may lead to possible mimetic drug candidates. The selected phage may also prove useful in vaccine development.

The idea of developing an 'epitope library' (Parmley and Smith, 1988) was inspired by the work of Geysen *et al.* (1986) on synthetic mimotopes. In this work, peptide mixtures which were synthesised on plastic pins were used to bind antibodies, and this led to the delineation of a peptide which mimicked a discontinuous antigenic determinant (Geysen *et al.*, 1986). This peptide was termed a mimotope, and synthetic mimotope strategy was recognised as having the potential for discovery of ligands for antibodies whose specificity was unknown.

The work was developed by Smith (1985) by incorporation of synthesised oligonucleotides into the coding region of the coat proteins of the filamentous bacteriophage fd tet, such that a fusion peptide was expressed in one of the coat proteins. The related phage m13 and f1 have also been adapted for phage library purposes (McLafferty *et al.*, 1993; Makowski., 1993; Felici *et al.*, 1991; Luzzago *et al.*, 1993).

1.4.2 Replication cycle of ff phage

The bacteriophage used in this project were derivatives of the filamentous phage fd, the structure of which is shown in Figure 2. The phage fd genome consists of a circular, single strand of DNA of 6408 nucleotides which is packaged in a protein sheath. The

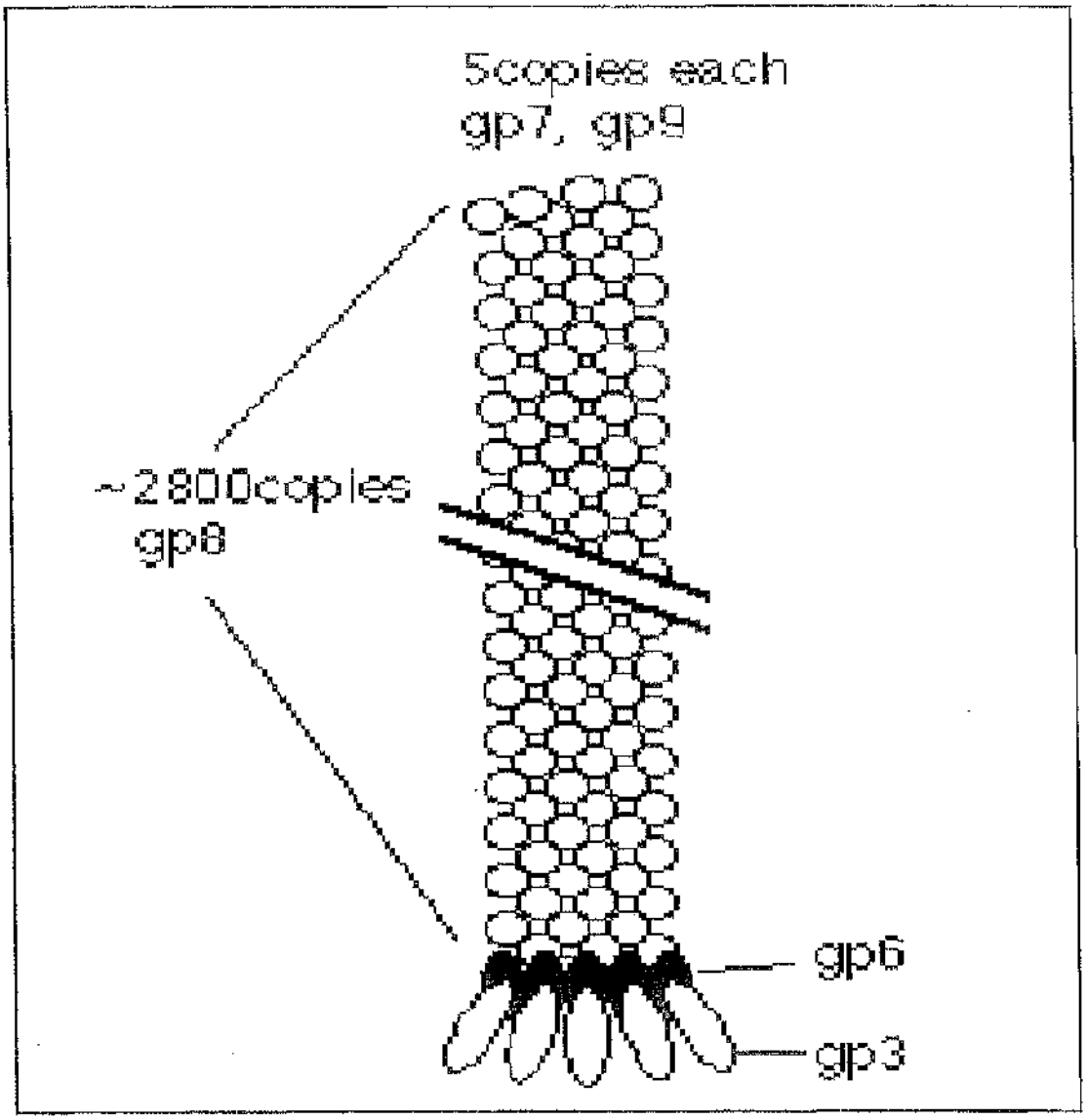


Figure 2. Structural organisation of the inoviruses fd, m13 and f1. Adapted from Kishchenko *et al.* (1994).

majority of this protein is comprised of the major coat protein of gene viii, called gp8 or gpVIII . Four other proteins are also present in the virion, the distal end being capped with a "plug" of 5 copies of gp7 and gp9, and the proximal end being capped with 5 copies of gp6 that subsequently bind 5 copies of gp3 to the phage structure. The N-terminal portion of gene iii is involved in attachment to the F-pilus and initiating infection (Pratt *et al.*, 1969; Gray *et al.*, 1981). Other proteins, not mentioned here, are also involved in the infection process.

Once inside the cytoplasm, the single-stranded DNA (the + strand) is converted to a double-stranded replicative form by the combined actions of RNA polymerase, which synthesizes a specific primer, and DNA polymerase III holoenzyme, in the presence of the *Escherichia coli* single-stranded DNA binding protein. The phage-encoded gene II protein makes a nick in the plus strand and elongation proceeds at the 3' hydroxyl end until a new (+) strand is formed (reviewed by Model and Russel, 1988). The new strands can either act as templates for complementary strand synthesis or can be targeted by the gene V protein which delivers the ssDNA for production and export of new virion progeny.

1.4.3 Development of the phage fd 'tet'

Bacteriophage fd was genetically manipulated by Zacher *et al.* (1979) to produce the filamentous phage cloning vector fd 'tet', which was possible once the entire sequence of fd phage had been established (Beck *et al.*, 1978; Schaller *et al.*, 1978). The fd 'tet' vector was created by inserting the 2.8 kbp *BglII* fragment from Tn*10* into the BamHI site, near the origin of replication of phage fd. The Tn*10* fragment conferred the trait of tetracycline resistance into the intergenic region of the fd phage genome. A diagram of the genetic map of fd phage plus the additional Tn*10* fragment is shown in Figure 3. The structure of the phage changes by becoming longer due to the extra DNA which must be packaged into the final virion. This is achieved by adding an extra molecule of protein viii for every 2.3 nucleotides added (Kishchenko *et al.*, 1994), such that the phage genome is extended by 2.8 kbp and the capsid contains an extra 1217 copies of coat protein VIII.

1.4.4 The Fuse5 vector

Bacteriophage fd 'tet' was further manipulated by Scott and Smith (1990) to produce the 'fuse' series of vectors required for the insertion of degenerate oligonucleotide sequences. The Fuse5 vector (Scott & Smith 1990) used in this project makes use of the coat protein gp3 which has a surface exposed N-terminal domain that tolerates foreign peptide inserts well (Smith, 1993). This vector was created by incorporation

of the insert shown in Figure 4 (containing a 14 bp 'stuffer region'), into gene iii of fd 'tet' (co-ordinates 2258 to 2263 of original fd phage; Beck *et al.*, 1978) with the removal of 4 bp.

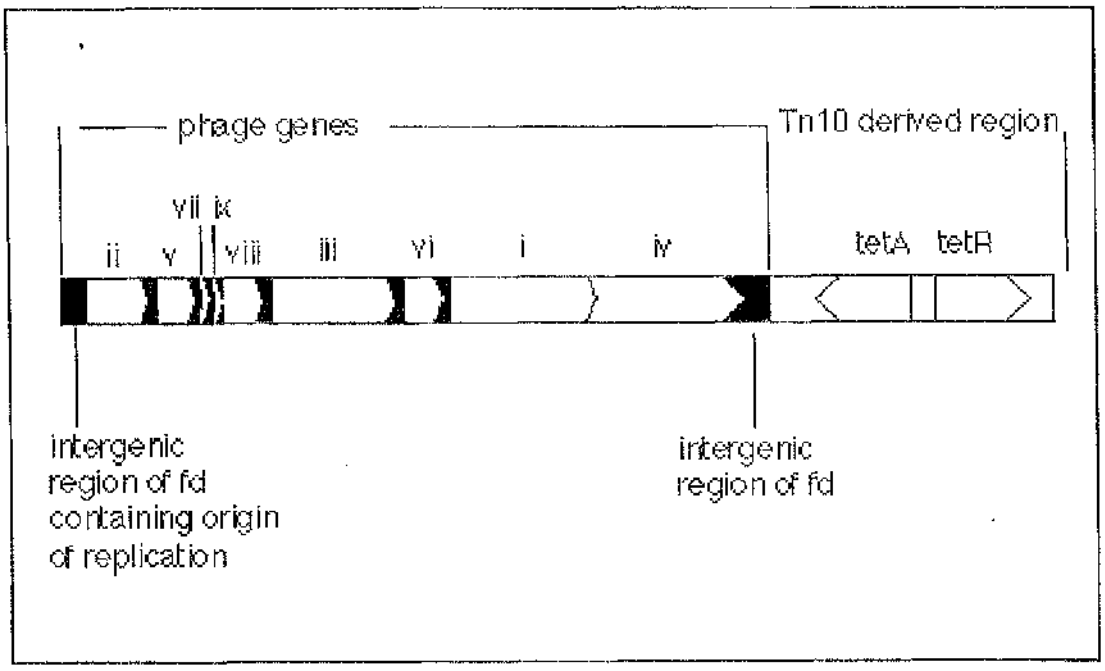


Figure 3. Genetic representation of phage fd 'tet'.
The location is shown of the Tn*10* fragment, which carries the tetracycline resistance gene, within the wild type fd inovirus. Adapted from Crissman and Smith (1984).

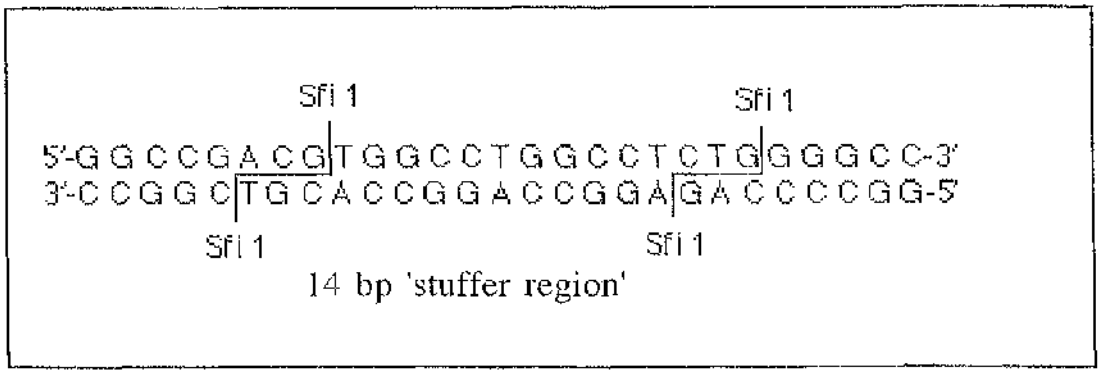


Figure 4. Fusc5 RF insert
Within the insert are *Sfi*I restriction sites. Digestion with *Sfi* I creates three base long, sticky ends and removes a 14 bp region known as a 'stuffer region' which allows for the insertion of the degenerate *Bgl*I (Figure. 5).

This insert is located just downstream of the eighteen amino acid (aa) signal peptide sequence and disturbs the reading frame. This prevents the synthesis of gene III coat protein A (gp3) rendering the phage unable to infect *E. coli* , as gp3 is required by the phage for binding to the F-pilus of male *E. coli* and initiating infection of the cell (Boeke and Model, 1982, Model and Russel, 1988). However, fuse5 can still be propagated as a tetracycline-resistant plasmid independently of infection (Hanahan, 1983; Scott and Smith, 1990).

1.4.5 Production of a 6 amino acid peptide phage display library in Gene III protein (gp3 coat protein)

The six amino acid phage library was made by ligating a degenerate 33 bp *Bgl* I fragment into the previously cleaved *Sfi*I site of fuse5 (Figure 5, with the consequent release of the 14 bp stuffer region (Figure 4).

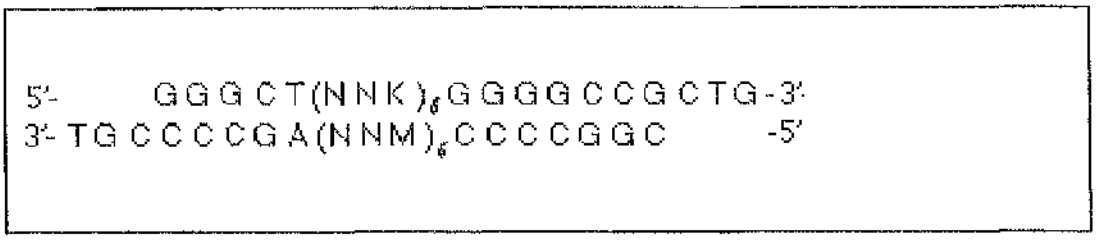


Figure 5. Degenerate *BglI* fragment incorporated into fuse5.

Insertion of the 33 bp *BglI* fragment restores the reading frame of gene III and when transfected into *E.coli* via electroporation (Scott and Smith, 1990) the progeny produce functional gene 3 protein and hence regain their infectivity.

The *BglI* fragment contain the degenerate coding sequence shown as $(NNK)_6$ in Figure 5, with N representing an equal mixture of the deoxynucleotides T, C, G and A during the random synthesis of the oligonucleotide and K representing an equal mixture of G and T. This provides coding flexibility for 32 codons, including one stop codon, giving a ratio of between 1 and 3 codons per amino acid and a possible 10^8 different nucleotide sequences. The final phage peptide library contains foreign peptide inserts of 6 amino acid with varying sequences in the coat protein gp3.

1.4.6 Other phage libraries

The previously discussed phage peptide library was made by insertion of foreign peptide inserts of 6 amino acids into coat protein gp3. Other libraries can be made by incorporation of larger inserts into gene III, e.g., as described for the 15 amino acid inserts in fuse5 vector (Scott and Smith, 1990). This library was also used in this project.

Other phage libraries can be made by inserting variable peptide inserts into the exposed N-terminal domain of the major coat protein VIII (variously named pVIII, gpVIII, gVIIIp, p8, gp8 or g8p) which tolerates foreign peptide inserts (Smith, 1993).

Other vectors carrying additional copies of gene III or VIII have been developed to allow greater stability and function of the phage with larger variable regions being inserted. The coat protein of gene viii can only tolerate insertions of 5 to 6 extra amino acids (Smith, 1993), but this can be increased by having two copies of gene viii, one being the wild type and the other an introduced copy containing the oligonucleotide library. This produces progeny virions that contains a mixture of wild type gp8 and gp8 protein displaying peptide inserts. The insert-bearing recombinant can be introduced either directly into an intergenic region of the phage, or by proxy using a phagemid which carries the insert-bearing recombinant. When grown in the same cell as fd-tet phage, the progeny of the phage contains both wild type protein from its own DNA and recombinant protein from the phagemid. This system can also be applied to gene III. Figure 6 demonstrates the range of phage display vectors currently available using f1, fd and m13 filamentous bacteriophage.

1.4.6 Constrained libraries

Constrained libraries differ from other libraries in that instead of the insert having non-interfering small amino acids such as glycine at each end, they have cysteine residues at each end. This has the purpose of forcing the insert into a loop as the cysteine-cysteine disulphide bonding occurs. This technique was used by McLafferty *et al.* (1993), in displaying disulphide-constrained peptides. Another example is provided by McConnell and Hoess (1994; 1995).

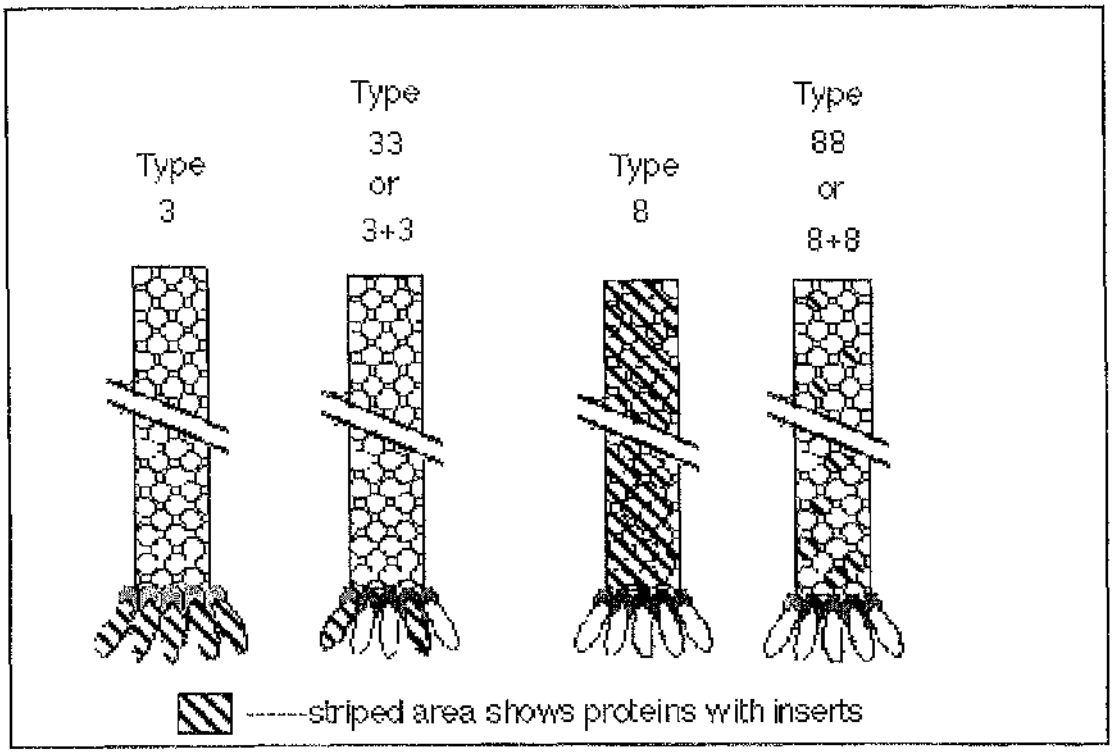


Figure 6. Classification of phage display vectors.
This diagram indicates the various phage vectors available using f1, fd and m13 filamentous bacteriophage. The striped areas indicate the proteins which contain inserts.

1.4.7 Recent applications of phage peptide display technology

Phage peptide display technology has recently been used successfully for displaying immunoglobulin variable domains (McCafferty *et al.*, 1990), alkaline phosphatase (McCafferty *et al.*, 1991), an immunogenic region of the HIV virus (Tsunetsuga-Yokato *et al.*, 1991), peptide sequences from the V3 loop of gp120 from HIV-1 strain MN (Veronese *et al* ., 1994), and pancreatic trypsin inhibitor (Roberts *et al.*, 1992). Phage peptide display technology has also been used to screen against various antibodies such as anti-β-endorphin mAb 3-E7 (Cwirla *et al.*, 1990).

OBJECTS OF RESEARCH

The aim of this project was to investigate whether bacteriophage displayed-peptide libraries system could be used to identify peptide mimotopes which mimic epitopes of polysaccharides antigens of *Aeromonas salmonicida*. The study was divided into two main sections. The two antigens chosen were the capsular polysaccharide (CPS), for which a polyvalent rabbit antiserum was available, and the lipopolysaccharide, for which a mouse monoclonal antibody was available. Both the 6-mer and 15-mer bacteriophage libraries, displayed in protein PIII (Smith 1990), were available for use.

Phage isolated by biopanning were to be compared by ELISA for their binding to anti-CPS and anti LPS antisera and the insert sequences compared to determine whether particular motifs were present in the inserts.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Unless otherwise specified all chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

2.1.2 Bacteriophage-displayed peptide libraries

The primary (type3) 6-mer peptide phage library (Scott and Smith, 1990), which contained 2 x 10^8 primary clones, was provided by Professor George P. Smith (Division of Biological Sciences, University of Missouri). The (type 88) 15-mer library was produced and provided by Sam Choukri of Smith's laboratory and contained 2 x 10^9 primary clones.

2.2 Amplification of the primary bacteriophage peptide library

The method was as described by Smith (1993) with some modifications.

2.2.1 Transduction and propagation of 6-mer phage library in *Escherichia coli* K91kan

A 10 ml overnight culture of *E. coli* K91kan in LB broth (Appendix) with 100 µg/ml kanamycin was shaken at 37°C overnight and 1 ml was used to inoculate each of two 250 ml dimpled flasks containing 99 ml Terrific broth (Appendix). The cultures were shaken at 150 r.p.m. at 37°C until the optical density at 600nm of a 1 in 10 dilution of the cultures reached 0.2. At this point the shaker speed was reduced to 10 r.p.m. for 5 min prior to the addition of 50 µl of the stock 6-mer bacteriophage library, titre approximately 10^{14} transducing units (TU)/ml, into flask A and 10 µl into flask B. The cultures were shaken for a further 15 min to allow infection to occur and the transformed cultures were then transferred to 2L flasks containing 1L of LB broth containing 0.22 µg/ml tetracycline. The flasks were shaken vigorously for 35 min at 37°C before addition of more tetracycline to a final concentration of 18.4 µg/ml. LB agar plates containing kanamycin (100 µg/ml) and tetracycline (40 µg/ml) were used to analyse 50 µl samples of transformed cells and untransformed cells. The plates and flask cultures were incubated at 37°C overnight.

2.2.2 Purification of phage

The amplified phage-infected *E. coli* cultures were centrifuged at 4300 x g for 10 min at 4°C, the supernatants re-centrifuged at 10800 x g for 10 min at 4°C and the final supernatants then transferred to three fresh 500 ml centrifuge tubes. After addition of 0.15 vol PEG/NaCl solution (Appendix), the tubes were inverted approximately 100 times to ensure complete mixing. PEG/NaCl precipitation was allowed to continue for 4 h (or overnight) on ice at 4°C. The tubes were then centrifuged at 10800 x g for 40 min at 4°C; after discarding the supernatant the tubes were recentrifuged and the residual supernatants discarded.

Each pellet was dissolved in 10 ml TBS by shaking for 30 min, and the phage solutions were centrifuged briefly to collect them at the bottom of the tubes; they were then transferred to two Oak Ridge centrifuge tubes (A and B). The collected phage suspensions were centrifuged at 27000 x g for 10 min, the supernatants transferred to fresh centrifuge tubes containing 4.5 ml PEG/NaCl solution and the phage incubated on ice for 1 h. The phage suspensions were again centrifuged at 27000 x g for 10 min and the supernatants discarded. The final phage pellets were each dissolved in 1 ml of TBS buffer (Appendix) by shaking for 1 h. The tube was then vortexed to ensure complete resuspension of the pellet and the liquid was recentrifuged at 12000 x g for 10 min, to remove any particulate material before being transferred to an Eppendorf tube for storage at 4°C.

2.2.3 Quantification of amplified phage

The above procedure (Transduction and propagation of 6-mer phage library) was also used for quantification of amplified phage. Samples of 50 µl were removed from the culture and plated on LB agar plates containing kanamycin (100 µg/ml) and tetracycline (40 µg/ml). The plates were cultured at 37 °C overnight.

2.2.4 Repeat amplification of stock phage

After removing a sample from culture B for quantification as described above the remaining culture was incubated at 37°C overnight to allow further amplification of the phage in culture B, and purification (see 'Purification of phage').

2.3 Biotinylation of antiserum

2.3.1 Labelling of antibody with biotin

The absorbance of an immunoglobulin G solution of 1 mg/ml is approximately 1.4 at 280 nm (Kabat and Meyer, 1964) and this relationship was used to establish the approximate protein concentration of an immunoglobulin fraction of a polyclonal antiserum to the capsular polysaccharide (CPS) of *A. salmonicida* (kindly provided by Professor F. Congregado, University of Barcelona). The antiserum was then diluted with 0.1 M sodium phosphate buffer to a final immunoglobulin concentration of 7 mg/ml (n.b. the protein concentration recommended by Smith (1993) was 10 mg/ml).

The method for biotinylation of antiserum was provided by the manufacturer of the biotinylation kit (Sigma) and this is summarised below. The contents of one vial of biotinamidocaproate-N-hydroxy-sulfosuccinimide ester (BAC-sulfoNHS) were dissolved in 30 µl dimethylsulphoxide, 0.1 ml of 0.1 M sodium phosphate was added to give a final volume of 0.5 ml, and the mixture was vortexed thoroughly. This process provided a 10 mg/ml Bac-sulfoNHS solution. Immediately, 38 µl of this BAC-sulfoNHS solution was added to 1 ml of the diluted anti-CPS antibody and mixed gently. The mixture was then shaken gently for 30 min at room temperature.

2.3.2 Isolation of labelled protein

A gel filtration column (Sephadex G-25, bed volume 9.1 ml) was equilibrated with 6 x 5 ml volumes of 0.01 M PBS buffer. The reaction mixture was added to the column and the flow-through material collected as fraction one. The column was then eluted with 9 ml of PBS buffer (0.01 M) and 9 x 1 ml fractions were subsequently collected. For each fraction the absorbance at 280nm was measured and appropriate fractions were pooled.

2.3.3 Determination of biotin/protein ratio

Method B of the manufacturer's protocol was followed, in which 0.1 ml of the pooled biotinylated protein fraction was mixed with 0.9 ml 0.01M phosphate buffer (PB) and

the absorbance recorded at 280 nm in a 1 cm path length quartz cuvette. This was the 'protein sample'.

Lyophilised pronase was reconstituted with 1 ml de-ionised water, and 10µl of the solution was added to 0.1 ml of the pooled biotinylated anti-CPS fraction. The mixture, termed the 'biotin' sample, was incubated for 1.5 h at 37°C.

The lyophilised avidin was reconstituted with 19.4 ml 0.01M PB and 3.2 ml of this solution was mixed with 0.1 ml of 10 mM 4'-hydroxyazobenzene-2-carboxylic acid (HABA) solution; the absorbance of the avidin-HABA mixture was recorded at 500 nm using 0.01M PB as a blank solution.

A mixture of 0.9 ml avidin-HABA solution and 0.1 ml of the 'biotin sample' was then prepared, together with a control mixture containing 0.9 ml avidin-HABA and 0.1 ml PB. The absorbance of both samples was recorded at 500 nm, and the biotin/protein ratio was calculated as shown below.

2.3.3.1 Protein concentration

From the optical density at 280nm of a 1/10 dilution of the immunoglobulin solution,

$$\text{protein concentration (mg/ml)} = \frac{OD_{280} \times 10}{1.4}$$

$$\text{nmols IgG /ml} = \frac{\text{mg protein/ml} \times 10^6}{150000}$$

assuming an average molecular weight for IgG of 150000. The factor of 10^6 is for conversion from moles/L to nmoles/ml.

2.3.3.2 Biotin concentration

The optical density of the avidin -HABA solution at 500 nm should be close to 1.0.

The corrected OD_{500} value was determined by subtracting the avidin-HABA / biotin value from the control mixture value.

$$\text{Biotin concentration (nmols/ml)} = \frac{\text{Corrected } OD_{500}}{E_{500}} \times 10 \times 10^6$$

where E_{500} is the HABA/avidin extinction coefficient, i.e. the A_{500} of a 1M biotin solution (= 34,000), 10 is the dilution factor, and 10^6 is for conversion of moles/L to nmoles/ml.

2.3.3.3 Biotin/protein ratio

The concentration of biotin molecules (nmols/ml) divided by the concentration of antibody molecules (nmols/ml) gives the ratio of biotin molecules per immunoglobulin molecule.

2.4 Biopanning

The following is a general description of the biopanning method and should be read in conjunction with the flow diagrams for each experiment.

2.4.1 Coating of Maxi-sorp tubes with streptavidin.

Streptavidin solution, 10 µl of a 1 mg/ml aqueous solution, was added to a Maxisorp tube (Nunc) containing 900 µl of sterile H_2O and 100 µl of filtered 1M $NaHCO_3$ solution. The tube was sealed with parafilm and shaken gently overnight at 4°C. The liquid was discarded and replaced immediately with blocking solution (Appendix). After 1 h the blocking solution was removed and the tube was washed rapidly six times with TBS/Tween solution (Appendix). To prevent the tube from becoming dry, the next stage was initiated immediately.

2.4.2 Reaction of bound streptavidin with biotinylated ligate

TBS/Tween solution containing 1 mg/ml BSA (400 µl) was pipetted into the freshly prepared streptavidin-coated Maxi-sorp tube and the biotinylated ligate was then added. Different volumes of biotinylated ligate, 10 µl and 50 µl, were added to two different tubes to determine the effect on phage recovery. Each tube was then sealed with parafilm and gently rocked at 4°C for 4 h before addition of 4 µl of a 10 mM filter-sterilised solution of biotin. The tubes were rocked gently for 1 h at 4°C to block any unbound streptavidin sites before washing the tubes 6 times with TBS/Tween; 400 µl of TBS/Tween and a further 4 µl of 10 mM biotin solution were added to each tube to ensure blocking of unbound streptavidin sites.

2.4.3 First round of biopanning

Each tube received 5 µl of the amplified library prior to being sealed with parafilm and shaken for 4 hr at 4°C. Precautions were taken to ensure that the phage solution did not come into contact with the tube above the level coated with streptavidin and blocked with BSA.

2.4.4 Removal of unbound phage and elution of bound phage

The tubes were emptied and washed 10 times with TBS/Tween, each tube being tapped down onto a clean paper towel after each wash to remove any residual unbound phage solution. Each tube then received 400 µl of glycine/HCl elution buffer (Appendix) and was rocked gently for 10 min at room temperature. The eluates were pipetted into 1.5 ml Eppendorf tubes containing 75 µl Tris/ HCl, pH 9.1 to raise the pH of the eluates to the range pH 7 - pH 8.5.

2.4.5 Amplification of eluates

A mixture of 100 µl of eluate and 100 µl *E. coli* K91kan cells, prepared as previously described, was shaken gently for 20 min at room temperature before transfer into 20 ml of prewarmed LB broth + 0.2 µg/ml tetracycline in a dimpled 250 ml flask and vigorous shaking for 45 min at 4°C.

[N.B. Titration of the input phage was also started at this point using the same batch of *E. coli* K91kan cells. Since effectiveness of transformation is strongly related to the bacterial growth phase, it was important to do both experiments in parallel with the same cells at the same time.]

After the 45 min incubation period, the concentration of tetracycline was increased to 18 µg/ml by addition of 20 µl of a 20 mg/ml tetracycline solution. At this point, 60 µl of the transformation mixture was removed and the remaining culture was grown overnight at 37°C. The 60 µl sample removed was used to provide 50 µl volumes of neat, 10^{-1} and 10^{-2} dilutions of transformed cells which were then spread onto LB plates containing kanamycin (100 µg/ml) and tetracycline (40 µg/ml). As a control, 50 µl of uninfected cells were also spread onto similar antibiotic-containing plates.

The amplified phage culture was transferred to a 50 ml Oak Ridge centrifuge tube and centrifuged at 5000 x g for 10 min. The supernatant was transferred to a fresh Oak

Ridge centrifuge tube and then centrifuged at 10000 x g for 10 min. The twice cleared supernatant was then mixed with 3 ml PEG/NaCl solution (Appendix) in a fresh Oak Ridge tube and the solution was mixed by inverting the tube 100 times. Precipitation of the phage was achieved by incubation at 4°C for 4 h (overnight incubation can increase precipitation of phage). The phage was pelleted by centrifugation at 10000 x g for 15 min, the supernatant was discarded and any residual supernatant was recentrifuged briefly and also discarded. The visible phage pellet was then dissolved in 1 ml TBS by pipetting and vortexing. The dissociated pellet was collected at the bottom of the tube by brief centrifugation and transferred to a 1.5 ml Eppendorf tube. The solution was then centrifuged at 15000 x g for 1 min to pellet and discard any insoluble matter. The phage pellet was transferred to a fresh 1.5 ml Eppendorf tube containing 150 µl of PEG/NaCl solution. The tube was inverted 100 times to mix thoroughly and then the phage was allowed to precipitate on ice for 1.5 h at 4°C.

The phage precipitate was centrifuged at 15000 x g for 10 min, the supernatant discarded and any residual supernatant recentrifuged briefly and also discarded. The final phage pellet was dissolved completely by pipetting and vortexing the pellet with 200 µl TBS.

2.4.6 Round 2 biopanning

2.4.6.1 Biopanning after pre-reacting phage with biotinylated ligate

Biotinylated immunoglobulin solution (4 µl of 5 µM solution) was added to 195 µl of first round amplified phage (from the previous step) to give a final concentration of 100 nM immunoglobulin (n.b. only a fraction of this was specific anti-CPS antibody). The phage and biotinylated ligate were allowed to react overnight at 4°C.

(During amplification of the 2nd round eluate the remaining 5 µl, of the 200 µl of amplified 1st round phage, was used to titrate the 'phage input' into the 2nd round.)

Following overnight incubation at 4°C, 800 µl of TBS/Tween was added to the reacted phage/antibody solution and the solution immediately pipetted into a freshly prepared streptavidin-coated maxi-sorp tube.

Removal of unbound phage and elution of bound phage were as described for round

one biopanning. Amplification of eluate and PEG-precipitation of phage were also done using the method described for round 1 biopanning.

2.4.7 Round 3 biopanning

Where appropriate, round 3 biopanning was done using the amplified eluate phage from round 2. The biotinylated immunoglobulin solution (anti-CPS) was mixed with the phage to a final concentration of 0.1 nM as opposed to 100 nM which was used in round 2. The lower molarity of biotinylated Ab was employed to select the phage binding most strongly to the ligand.

All other steps for round 3 were completed as described in round 2.

2.4.8 Analytical titration of input phage

This method was used to quantify the phage entering each round of biopanning.

The phage solution was diluted in TBS/gelatin (0.1 g/100 ml) solution by serial 10-fold dilution up to 10^{-11}. A 10 µl aliquot of each phage dilution and 10 µl of *E. coli* K91kan cells, freshly prepared in Terrific broth, were pipetted into 1.5 ml Eppendorf tubes. The mixtures were allowed to incubate for 10 min at room temperature, after which 1 ml of LB broth, + 0.2 µg/ml tetracycline, was added to each tube. The lids on the Eppendorf tubes were shut tightly before placing them on their sides on a shaker for 30 min at 37°C. Samples of 50 µl of the infected cultures were removed and spread onto tetracycline/kanamycin plates (40 and 100 µg/ml, respectively) which were incubated overnight at 37°C.

2.4.9 Biopanning a 6-mer phage library with anti-CPS antiserum

The previously described method was applied to the first set of three rounds of biopanning with CPS Ab as depicted by Figure 7. A repeat of this experiment differed in the second round where CPS (120 µg/400 µl) was used as the eluant rather than glycine/HCl buffer. This strategy was used in an attempt to enhance the selection of phage that mimic CPS. This method is depicted in Figure 8. The experiment led to the isolation of 40 phage clones which were eluted by glycine/HCl in the first round and CPS in the second round. The phage were subsequently purified for ELISA and 17 subsequently sequenced.

Figure 7. Protocol for three rounds of biopanning using biotinylated anti-CPS antiserum and 6-mer phage library. All three rounds were eluted with glycine HCl, pH 2.2. The initial input of biotinylated ligate was 10 ml (5 mM, i.e. 50 pmoles/tube). Note: for simplicity washing steps are not shown.

Experiment one.

Round One

50 pmoles anti-CPS IgG fraction + 6-mer phage library ----> bound phage eluted in 475 µl pH 2.2 buffer ----> 100 µl eluate amplified with Ecoli K91 kan ----> Phage recovered in 200 µl buffer (to round 2)

Round Two

1 pmole anti-CPS IgG fraction + 196 µl round one phage ----> " ----> " ----> " (to round 3)

Round Three

1 fmole anti-CPS IgG fraction + 196 µl round two phage ----> 1st elution with 475 µl pH 2.2 buffer

2nd elution with 475 µl pH 2.2 buffer

}Taken no further.

Figure 8. Protocol for two rounds of biopanning using biotinylated anti-CPS antiserum and 6-mer phage library. In the first round bound phage were eluted with glycine HCl, pH 2.2 and in the second round with CPS (120 µg/400 µl). Of the 40 phage selected for ELISA, 17 were subsequently sequenced. The initial input of biotinylated ligate was 50 pmoles.

Experiment Two

Round One

50 pmoles anti-CPS IgG fraction + 6-mer phage library. ----> bound phage eluted in 475 µl pH 2.2 buffer. ----> 4 x 100 µl eluate amplified with Ecoli K91 kan. ----> Each set of amplified phage was recovered in 200 µl buffer and pooled to give 800 µl total (to round 2).

Round Two

In five tubes (A-E) was added 0.5 pmole anti-CPS IgG fraction + 98 µl round one phage ----> Bound phage eluted (A&B) in 475 µl of CPS (300µg/ml), (C&D) in 475 µl TBS and E in 475 µl pH 2.2 buffer. ----> From Tube A, 40 phage were selected of which 17 were sequenced. B-E were taken no further.

The second round eluate from the above biopanning experiment was further amplified as shown in Figure 9.

2.4.10 Biopanning with a 6-mer phage library and biotinylated monoclonal antibody to *Aeromonas salmonicida* LPS

2.4.10.1 Processing and biotinylation of anti-LPS monoclonal antibody

A monoclonal antibody (MAb) against the lipopolysacharide of *A. salmonicida* , which had been prepared by Mrs. Julia Dunlop during a previous project, was used. It was termed F9 clone 16 and was supplied as 45 ml of tissue culture (hybridoma) supernatant fluid which had been purified by protein G (Pharmacia) affinity chromatography. The solution was concentrated by dialyis against PEG 20000 for 4 h, after which time the contents of the dialysis sac, 1.75 ml, were recovered and the tubing washed out using a further 0.5 ml saline buffer. The total volume of 2.25 ml of concentrated anti-LPS MAb contained 4.2 mg/ml protein and it was biotinylated as previously described (Section 2.3).

2.4.10.2 Biopanning of the 6-mer phage library with biotinylated anti-LPS monoclonal antibody.

Biopanning was carried out as described above (Sections 2.4.1 to 2.4.7) and the protocol is summarised in Figure 10. At various stages of the biopanning process, colonies were selected from the plates and the phage amplified and purified (see previously described method). The selected phage were assayed by ELISA using method C (Section 2.6.4).

Figure 9. Two rounds of biopanning using biotinylated anti-CPS antiserum and 6-mer phage library.
Repeat amplification of second round eluate and continuation to third round biopanning using CPS (120 mg/400 ml) as the eluant.. Of the 90 phage selected for ELISA, 22 were subsequently sequenced.

Continuation of round 2 of experiment two

Round Two

100 µl eluate from tubes A, B & E were amplified with *Ecoli* K91 kan ----> Taken through to round 3.

Round Three

3 tubes (A, B & E) x 0.5 fmole anti-CPS IgG fraction + 98 µl Round 2 ----> A&B bound phage eluted with 475 µl CPS (300µg/ml) and tube phage E bound phage eluted in 475 µl pH 2.2 buffer. ----> Tubes A & B produced phage 41 to 80 and 1 to 40 respectively and Tube E gave phage 81 to 90. These phage were subsequently assayed with ELISA or the insert DNA sequence determined.

Figure 10. Protocol for three rounds of biopanning using biotinylated anti-LPS MAb and a 6-mer phage library. All three rounds were eluted with glycine HCl, pH 2.2. After the first elution in round one (producing A1 & B1), another elution was carried out on tubes A1 & B1 (leading to C1 & D1). Selected phage for ELISA and insert sequencing are shown.

Round One

Tubes A1 and B1 received 50 pmoles anti-LPS IgG fraction + 6-mer phage library ----> bound phage eluted in 475 µl pH 2.2 buffer for each tube (A1, B1) This was repeated to obtain a second elution for each tube (C1, D1). ----> 100 µl eluate amplified with *E.coli* K91 kan. ----> Phage recovered in 200 µl buffer A1 gave phage 1 to 6 D1 gave phage 7 to 10.

Round Two

Tubes A to D received 1 pmole anti-LPS IgG fraction + 196 µl round one phage ----> bound phage eluted in 475 µl pH 2.2 buffer for each tube (A2, B2, C2. & D2) ----> 100 µl eluate amplified with Ecoli K91 kan. ----> Phage recovered in 200 µl buffer (to round 3). A2 gave phage 11 to 15. D2 gave phage 16 to 20.

Round Three

Tubes A to D received 1 fmole anti-LPS IgG fraction + 196 µl round two phage ----> bound phage eluted in 475 µl pH 2.2 buffer for each tube (A3, B3, C3. & D3) ----> A3 gave phage 21 to 51. B3 gave phage 52 to 65. C3 gave phage 66 to 76. D3 gave phage 77 to 96.

2.4.11 Biopanning with a 15-mer peptide library and anti-LPS monoclonal antibody

2.4.11.1 Centricon filtration of eluate from round one biopanning

Biopanning with the 15-mer phage library was carried out as for the 6 mer library. In the first round of biopanning 10 µl of biotinylated anti-LPS MAb was used along with 5 µl of 15-mer stock phage library. Duplicate tubes were used in Round 1 and the bound phage was eluted with glycine/HCl buffer. One of the two eluates from Round one was concentrated using a 30 kDa Centricon filter and the recovered phage suspension amplified. The resulting 200 µl amplified phage suspension was divided between two streptavidin coated Maxi-sorp tubes for the second round of biopanning.

2.4.11.2 Biopanning Rounds two and three

The following flow diagram (Figure 11) indicates all three rounds of biopanning, including round one described above. At the end of round three , 40 clones were selected and the phage amplified and purified as previously described. These clones were assayed by ELISA and the insert DNA sequences of 5 phage derived from tube 12 were determined.

2.5 Propagation and processing of phage on a small scale

Individual colonies were picked from the plates and cultured separately. Tubes (18 mm x 150 mm) containing 1.7 ml LB broth + 20 µg/ml tetracycline were inoculated with individual colonies and incubated at 37°C overnight. The cultures were transferred to 1.5 ml Eppendorf tubes and the cells pelleted by centrifugation at 20000 x g for 10 min. A 1 ml sample of supernatant was then transferred to a fresh Eppendorf tube containing 1.5 ml PEG/NaCl solution and tubes were inverted 100 times to mix the solution thoroughly. After incubation on ice for 4 h the precipitate was collected by centrifugation at 20000 x g for 15 min, the supernatant discarded, and the phage pellet was dissolved in 500 µl of TBS by vigorous vortexing.

Figure 11. Three rounds of biopanning using biotinylated anti-LPS monoclonal antibody and a 15-mer phage library. This diagram shows three rounds of biopanning. In round two, both tubes received a first and second wash using either LPS or glycine/HCl buffer as the eluant and in round three each second round eluate was divided into two tubes with one being eluted with glycine/HCl and the other eluted with LPS solution (10 µg/ml). Out of the forty phage selected for ELISA, five were subsequently sequenced (No. 36-40).

Round One

50 pmoles anti-LPS IgG fraction + 15-mer phage library ----> bound phage eluted in 475 µl pH 2.2 buffer and concentrated to 100 µl using a centricon filter ----> 100 µl eluate amplified with *E. coli* K91 kan ----> Phage recovered in 200 µl buffer (to round 2)

Round Two

2 x 0.5 pmole anti-LPS IgG fraction + 196 µl round two phage ---->

Tube 1- 1st wash Acid elution
2nd wash LPS elution (32 µg/ml) ---->
Tube 2- 1st wash LPS elution (10 µg/ml)
2nd wash LPS elution (32 µg/ml)

100 µl eluate amplified with *E. coli* K91 kan ----> Phage recovered in 200 µl buffer Tubes 1 to 4. (to round 3)

Round Three

0.5 fmole anti-LPS IgG fraction + 98 µl round two phage
Tube 1 (proceeded to tubes 5 and 6)
Tube 2 (proceeded to tubes 7 and 8)
Tube 3 etc. etc.
A total of 8 tubes (Nos. 5 to 12)

----> bound phage in odd numbered tubes were eluted in 475 µl pH 2.2 buffer. ----> bound phage in even numbered tubes were eluted in 475 µl LPS (32 µg/ml)

Tube 5 gave phage 1 to 5.
Tube 6 gave phage 6 to 10.
Tube 7 gave phage 11 to 15.
Tube 8 gave phage 16 to 20.
Tube 9 gave phage 21 to 25.
Tube 10 gave phage 26 to 30.
Tube 11 gave phage 31 to 35.
Tube 12 gave phage 36 to 40.

2.6 ELISA Procedures

Various ELISA methods were used with each set of phage selected for analysis, with differences in the materials used to coat microtitre plates and in the detection systems. The main methods used are described below. Methods A and B were first applied to the CPS clones obtained from the previously described experiments.

2.6.1 Purification of phage for ELISA

The phage suspension (Section 2.5) was centrifuged for 1 min at 20000 x g to pellet any insoluble matter and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube containing 75 µl PEG/NaCl solution. After thorough mixing by inverting the tube 100 times the suspensions were incubated at 4°C for 4 h, then centrifuged at 20000 x g for 15 min, the supernatant fluid removed and any residual fluid removed after further centrifugation for 1 min. The pellet was dissolved in 0.15 M NaCl by vigorous vortexing; insoluble matter was pelleted by centrifugation for 1 min at 20000 x g, and the supernatant was transferred to a fresh Eppendorf tube which contained 11.1 µl 1M acetic acid. The fluid was mixed thoroughly followed by incubation for 10 min at room temperature, and an additional 10 min on ice, after which the phage were collected by centrifugation at 20000 x g for 30 min at 4°C and the supernatant discarded. Any residual supernatant fluid was discarded after brief centrifugation. The phage pellet was dissolved in 200 µl TBS (sometimes 500 µl) and insoluble matter was removed as described previously.

2.6.2 Method A

This method was used for selected phages from the second round of biopanning with polyclonal anti-CPS antiserum. The phage were eluted with glycine/HCl buffer in the first round and CPS (120 µg/ml) in the second round.

Flat bottomed 96-well ELISA plates were used for all experiments and each clone was assayed in duplicate. After dispensing 40 µl phage suspension per well the plate was covered and placed at 4°C overnight before rinsing each well by pipetting 200 µl blocking solution (see Appendix) into each well and removal with a Gilson pipette. Wells were then blocked with 350 µl of blocking solution overnight at 4°C.

The blocking solution was removed and the wells washed three times with TBS/Tween (0.5 % v/v Tween 20). Each well then received 35 µl biotinylated anti-CPS antibody (30 nM solution in TBS/Tween + BSA 1 mg/ml). The reaction was completed overnight at 4°C, after which the ligate solutions were removed and the wells washed seven times with TBS/Tween and once with TBS. Biotinylated anti-rabbit antibody solution (35 µl, 5 µg/ml) were added to each well and allowed to react at 4°C overnight. The plate was washed a further seven times with TBS/Tween and once with TBS. Each well received 85 µl Horseradish Peroxidase Avidin D (HRP-avidin; Vector laboratories, Cat. No. A-2004) (diluted in TBS/0.1% Tween) and the plate was incubated for 30 min at room temperature. The plate was then washed seven times with TBS/Tween and once with TBS. Finally each well received 85 µl of freshly prepared ABTS peroxidase substrate solution [2,2'-Azino-di-(3-Ethyl-Benzthiazoline sulphinate) 6] (Dynatech Laboratories) and after incubation for 1 h at room temperature the optical density of each well was measured at 405 nm at an equal time after the addition of the substrate. The readings were compared to the control readings at 492 nm but it was not deemed necessary to deduct these values, from the test values, since no abnormal readings were found at 492 nm (see Controls section below).

Appropriate controls were included for each step; where phage was omitted, an equal volume of TBS was used in it place. Other controls used were TBS instead of biotinylated antibody, biotinylated anti-rabbit antibody and avidin. Purified CPS was also added in place of phage at concentrations of 10, 1, 0.1 µg/ml. Stock phage was also assayed at various concentrations (neat, $^1/_3$, $^1/_{10}$, $^1/_{31}$, $^1/_{100}$ in TBS) in place of selected phage clones.

2.6.3 Method B

This method was used for analysis of selected clones from the second round of biopanning using polyclonal anti-CPS antibody. Initial steps and volumes used were as in Method A, except that only 40 µl HRP-avidin solution in TBS/0.1% Tween was used. The optical density of each well was read as described previously

2.6.4 Method C

This was used for phage selected from the 6-mer library in the three rounds of biopanning using biotinylated anti-LPS MAb. Phage from each clone were assayed in duplicate in 40 µl volumes as described in ELISA Method A (Section 2.6.2), except that biotinylated anit-LPS MAb (50 nM in TBS/Tween with BSA, 1 mg/ml) was used. After 15 h at 4°C the ligate solutions were aspirated from the wells, which were then washed ten times with TBS/Tween and once with TBS. Each well received 35 µl HRP-avidin solution (Vector Labs ABC reagent) diluted in TBS/0.1% Tween. The plate was incubated for 30 min at room temperature before being washed seven times with TBS/Tween and once with TBS. Finally, each well received 85 µl of freshly prepared ABTS solution and after reaction at room temperature the optical density of each well was read after 5 min and 60 min as described previously.

2.6.5 Effect of phage concentration on the ELISA response

Clones 1 and 2 (high binding phage) and clone 6 (low binding phage) were selected and serial dilutions (neat, $^1/_3$, $^1/_{10}$, $^1/_{31}$, $^1/_{100}$) were made in TBS. Each dilution was then assayed with the ELISA described in method B.

Clones 5, 18 and 28 (high binding phage) and clones 25 and 26 (low binding phage) were also assayed (6 wells per clone) at a dilution of 1/6 in TBS.

2.6.6 Competitive ELISA

The high binding phage 1, 5, 28, 40 and low binding phage 6 and 26 were re-amplified and purified for this experiment. Prior to the addition of the biotinylated antibody (method B) selected wells received 35 µl of CPS at five different concentrations (10, 3.2, 1, 0.32 and 0.1 µg/ml). Standard controls were applied (dilutions of stock phage, no avidin, no anti-CPS, and dilutions of CPS).

2.6.7 Binding properties of capsular polysaccharides to ELISA plates

The binding of CPS was tested at pH 5, 6, 7, 7.5, 8, 9, and concentrations of 320, 160, 80, 40, 20, 10, 2.5, and 0 µg/ml CPS (diluent was TBS with acetic acid or sodium bicarbonate solution for pH modification). The ELISA was performed using method B except that CPS was used in place of phage.

2.6.8 ELISA for phage

Phage suspensions were analysed by ELISA using HRP-conjugated anti-M13 antibody (Pharmacia, product No. 27-9402-01). The method was as described for anti-CPS except that the HRP-anti-phage antiserum was added in place of the anti-CPS. The reaction was allowed to proceed for 14 h at 4°C followed by 10 washes with TBS. After addition of substrate, the plate was incubated and the absorbance read as previously described.

2.7 Sequencing

2.7.1 Preparation of DNA samples

To 200 µl of phage from small scale propagation and purification was added 200 µl of phenol / chloroform (1:1 v/v). The solution was thoroughly mixed by vortexing, centrifuged for 15 min at 15000 rpm and the aqueous phase transferred to a fresh 1.5 ml Eppendorf tube which contained 250 µl TE buffer. This solution was mixed with 40 µl 3M sodium acetate solution, 1 ml ethanol was added with mixing and DNA was allowed to precipitate for 1 h at - 20°C. The precipitated DNA was pelleted by centrifugation for 30 min at 20000 x g. The supernatant fluid was aspirated from the tube, discarded and the DNA washed once with 1ml 70% ethanol (carefully applied down the centripetal wall of the tube). The tube was centrifuged at 20000 x g for 8 min and the supernatant discarded; the pellet was washed once more with 1ml 70% ethanol and the supernatant discarded as before. The final product was dissolved in 7 µl water.

2.7.2 DNA agarose gel electrophoresis

Gel preparation and sequencing procedure is described in the Appendix.

2.8 Electron Microscopy

From an overnight culture of uninfected *E. coli* K91kan cells a sample of 100 µl was mixed with 10 µl of formalin. After 1 h, 50 µl of the mixture was collected by centrifugation and washed 3 times with PBS to give a final volume of 50 µl. An overnight culture of phage-infected *E. coli* K91kan cells was processed similarly

The samples were analysed at the University of Glasgow IBLS Electron Microscopy unit with the assistance of Margaret Mullin using a Zeiss 902 transmission electron microscope.

3. RESULTS

3.1 Amplification of primary bacteriophage peptide library

When amplifying a primary bacteriophage peptide library it is necessary to calculate the concentrations of the phage used for infection and the phage recovered (see Methods section for explanation of calculations). Whilst good amplification can maintain an effective library, poor amplification can damage the effectiveness of a library by reducing the diversity of inserts available.

3.1.1 Growth of *E. coli* K91kan in broth culture

The host bacterium used for amplification of the filamentous bacteriophage fd tet peptide library was *E. coli* K91kan. The f-pilus structures on the surface of the bacterium are recognised by the minor coat protein III of the fd phage, leading to infection of the organism. For optimum replication of the phage it is essential that they are introduced into the *E. coli* culture when cells carry the greatest number of fully formed F-pili and hence are most susceptible to infection. According to Smith (1993) the best potential for infectivity occurs during late exponential growth when a 1/10 dilution of the *E. coli* K91kan culture reaches an optical density (OD) of 0.2 at 600nm.

Figure 12 shows duplicate growth curves of *E. coli* K91kan and demonstrates the consistency of growth of the organism when all other growth parameters remain constant. This proved useful when preparing cells for infectivity, and planning of experiments. From the results shown in Figure 12, an absorbance of 0.2 (at a 1/10 dilution) was reached within 4 h, and it was feasible to leave cultures to grow unattended for a period of 3 h, before regular monitoring until the appropriate absorbance was reached. This was at approximately 3h 40 min after establishing the culture.

3.1.2 Amplification

Two separate amplifications were done from the initial library (Experiments A and B), followed in Experiment C by further amplification of the product of Experiment B.

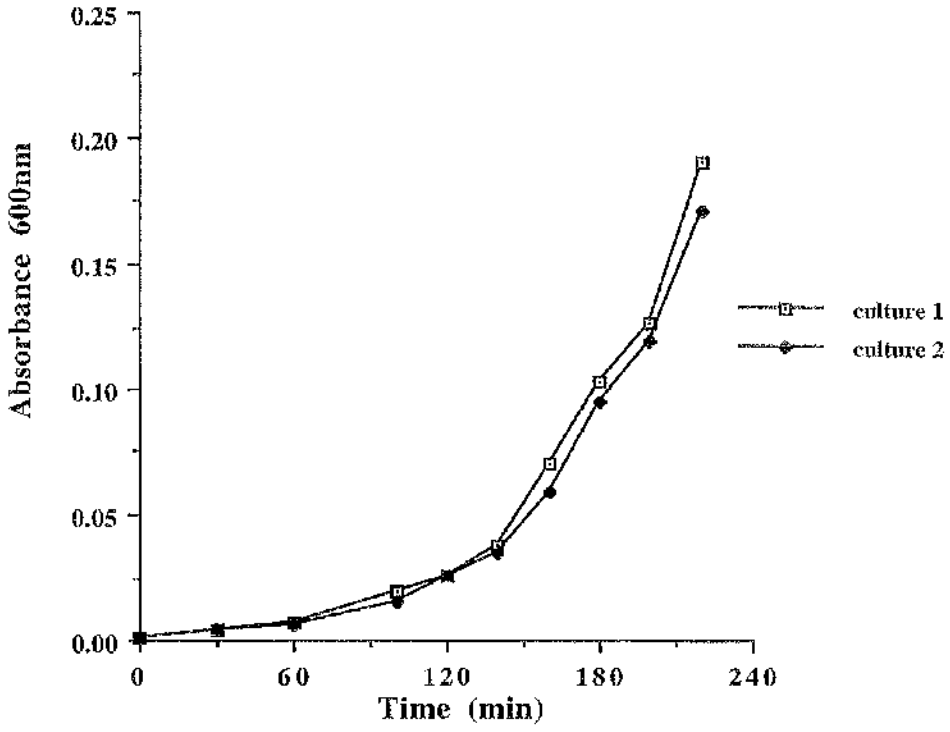


Figure 12. Growth curve for *E. coli* K91 kan in LB broth

Prewarmed LB broth, 100 ml in each of two 250 ml dimpled Erlenmeyer flasks, was inoculated with 1 ml of an overnight culture of *E. coli* K91Kan. The cultures were shaken at 200 oscillations per min at 37°C and the absorbance at 600nm of a 1/10 dilution of cultures measured at regular intervals for up to 3 h 40 min. Absorbances shown are of 1 in 10 dilutions of the cultures.

3.1.3 Experiment A

In the first amplification (Experiment A) 50µl of stock phage was used to infect *E. coli* K91kan, and the titre of the phage suspension was determined as 2.6 x 10^{10} TU/ml, corresponding to 7.15 x10^{11} virions/ml, assuming an efficiency of TU determination of 4 % (Smith, 1993). Thus, there were 3.6 x 10^{10} virions in the 50µl inoculum, and the number of phage recovered was 3.3 x 10^{10} virions in a volume of 1 ml. The amplification factor in this experiment was 0.9, i.e. a reduction in titre and thus an unsuccessful amplification step.

3.1.4 Experiment B

In this experiment 10 µl of phage (1.8 x 10^{10} virions) was used as inoculum for amplification; 1.1 x10^{11} virions were recovered in a volume of 1 ml, corresponding to an amplification factor of 6, despite a reduction in phage concentration from the starting level of 1.8 x10^{12} virion/ml to 1.1 x10^{11} virion/ml.

3.1.5 Experiment C

The phage from experiment B would be suitable for biopanning, however, it was considered that greater amplification should be achievable, and a repeat amplification was performed on the product of Experiment B. The 10 µl input phage contained 1.1 x10^{9} virions which yielded 8.25 x10^{11} virions in a volume of 1 ml.The amplification factor of 750 was acceptable and this phage stock was used in subsequent biopanning experiments.

3.2 Biotinylation of rabbit anti-CPS antiserum

An immunoglobulin fraction of rabbit anti-*A. salmonicida* CPS antiserum, prepared by ammonium sulphate precipitation, was diluted to a protein concentration of 7 mg/ml for labelling with biotin. After biotinylation the labelled protein was separated from free biotin by gel filtration. The elution profile from the column is shown in Figure 13. Fractions 4 to 7 were pooled together to give 4 ml of biotinylated antibody solution, calculated from A280nm measurement to contain 0.82 mg/ml protein (5.5 nmole IgG/ml).

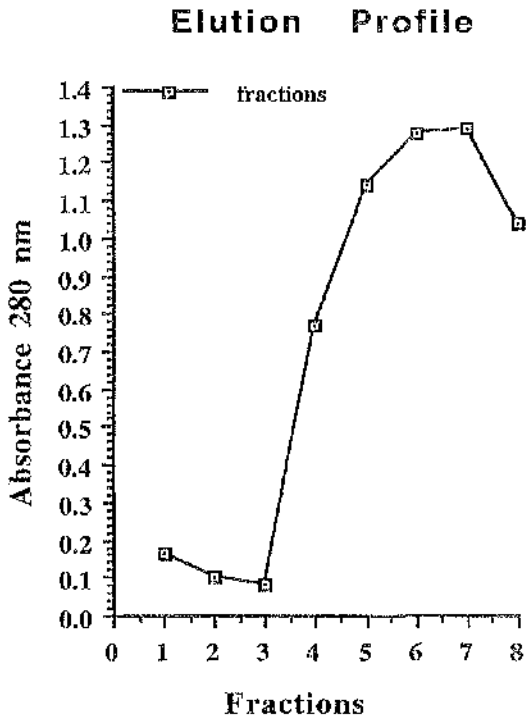


Figure 13. Separation of biotinylated anti-CPS immunoglobulin from free biotin by Sephadex G 25 chromatography.

One ml biotinylated immunoglobulin reaction mixture from rabbit antiserum to *A. salmonicida* CPS was applied to a column of Sephadex G25 Fractions of 1 ml were collected and the absorbance at 280nm measured. Fractions 4, 5, 6 and 7 were combined to give 4 ml of biotinylated antibody. Free biotin was eluted in subsequent fractions (not shown).

The concentration of biotin in the solution was determined to be 40.6 nmol/ml (see Methods section for details of calculations), giving a ratio of 7.4 biotin molecules per antibody molecule. Such a ratio is satisfactory to achieve adequate binding of antibody molecules to the streptavidin-coated tubes.

3.3 Biopanning with biotinylated rabbit-anti-CPS antiserum

A schematic diagram of the biopanning done in Experiments 1 and 2 with biotinylated polyvalent anti-CPS antiserum is shown in Figures 7 and 8. The quantities of phage applied and recovered in all experiments with anti-CPS antiserum and the 6-mer library are summarised in Table 2.

3.4 Analysis by ELISA of phage selected by biopanning with anti-CPS antiserum

3.4.1 Comparison by ELISA of the interaction with anti-CPS antiserum of phage selected by biopanning

Forty separate clones selected from the eluate of tube A (round 2) (see Table 2) were amplified, the phages purified and used for ELISA. The results (Figure 14; 39 clones shown) indicated a high background absorbance value for the control phage (the stock phage library); this was thought to be due to the use of a biotinylated second antibody (anti-rabbit IgG antiserum) as well as biotinylated first antibody (rabbit anti-CPS antiserum). The experiment was repeated omitting the biotinylated anti-rabbit IgG reagent to produce the results shown in Figure 15. The background absorbance of < 0.1 at 405nm was more satisfactory and gave greater discrimination between high and low binding phage compared to the results shown in Figure 14. With the exception of phage 40 (not tested in the first experiment) and phage 39, those phage which gave an absorbance value > 0.01 above background in Figure 15 gave an absorbance above background in Figure 14. This consistency suggests that there had probably been selection of phage with an affinity for anti-CPS antibody.

Table 2. Phage applied to and recovered from three rounds of biopanning with anti- CPS antiserum and 6-mer phage.

Experiment cycle	Biopanning	Biotinylated anti-CPS antiserum used	Phage applied/source	Elution buffer	Elution volume	Vol. eluate amplified (µl)	total virions applied	total virions recovered	recovery (%)	Use of clones
1	1	50 pmoles	10µl (library)	pH 2.2	475µl	100µl	1.7×10^{13}	1×10^{8}	5.8×10^{-4}	cycle 2
	2	1 pmole	196µl (cycle 1)	pH 2.2	475µl	100µl	1.7×10^{16}	8.3×10^{7}	5.8×10^{-4}	cycle 3
	3	1 fmole	196µl (cycle 2)	pH 2.2	475µl[1]	0[2]	2.7×10^{13}	5.6×10^{5}	2.1×10^{-5}	Taken
							2nd wash -	2.1×10^{6}	7.7×10^{-6}	no further
2	1	250 pmoles	(10µl library)	pH 2.2	475µl	4 x 100µl (3)	6.2×10^{11}	5.8×10^{7}	9.4×10^{-3}	
	2 a	0.5 pmoles	98µl (cycle 1)	CPS	475µl	100µl	3×10^{11}	3.6×10^{7}	0.012 (÷ 40 clones ELISA &	cycle 3 a selected for sequencing)
	2 b	0.5 pmoles	98µl (cycle 1)	CPS	475µl	100µl	3×10^{11}	7.2×10^{7}	0.024	cycle 3 b
	2 c	0.5 pmoles	98µl (cycle 1)	TBS	475µl	n.d.	3×10^{11}	7.2×10^{7}	0.024	
	2 d	0.5 pmoles	98µl (cycle 1)	TBS	475µl	n.d.	3×10^{11}	4.3×10^{7}	0.014	
	2 e	0.5 pmoles	98µl (cycle 1)	pH 2.2	475µl	100µl	3×10^{11}	4.6×10^{7}	0.015	cycle 3 e
3	3 a	0.5 fmoles	98µl (cycle 2a)	CPS	475µl	n.d.	1.3×10^{14}	1.4×10^{7}	1.0×10^{-5}	ELISA 41-80
	3 b	0.5 fmoles	98µl (cycle 2b)	CPS	475µl	n.d.	1.3×10^{14}	9×10^{6}	6.9×10^{-6}	ELISA 1-40
	3 e	0.5 fmoles	98µl (cycle 2e)	CPS	475µl	n.d	1.3×10^{14}	5.3×10^{5}	4.0×10^{-7}	ELISA 81-90

Figure 14. ELISA of 40 isolated phage selected by biopanning a 6-mer library with anti-CPS antiserum.

The ELISA reaction was terminated at 35 minutes.
The 1st round elution was with glycine/HCl and the 2nd round with CPS (120 μg/400 ml). The background reading is shown by the solid line. This ELISA involved an amplification step using biotinylated anti-rabbit antibody.

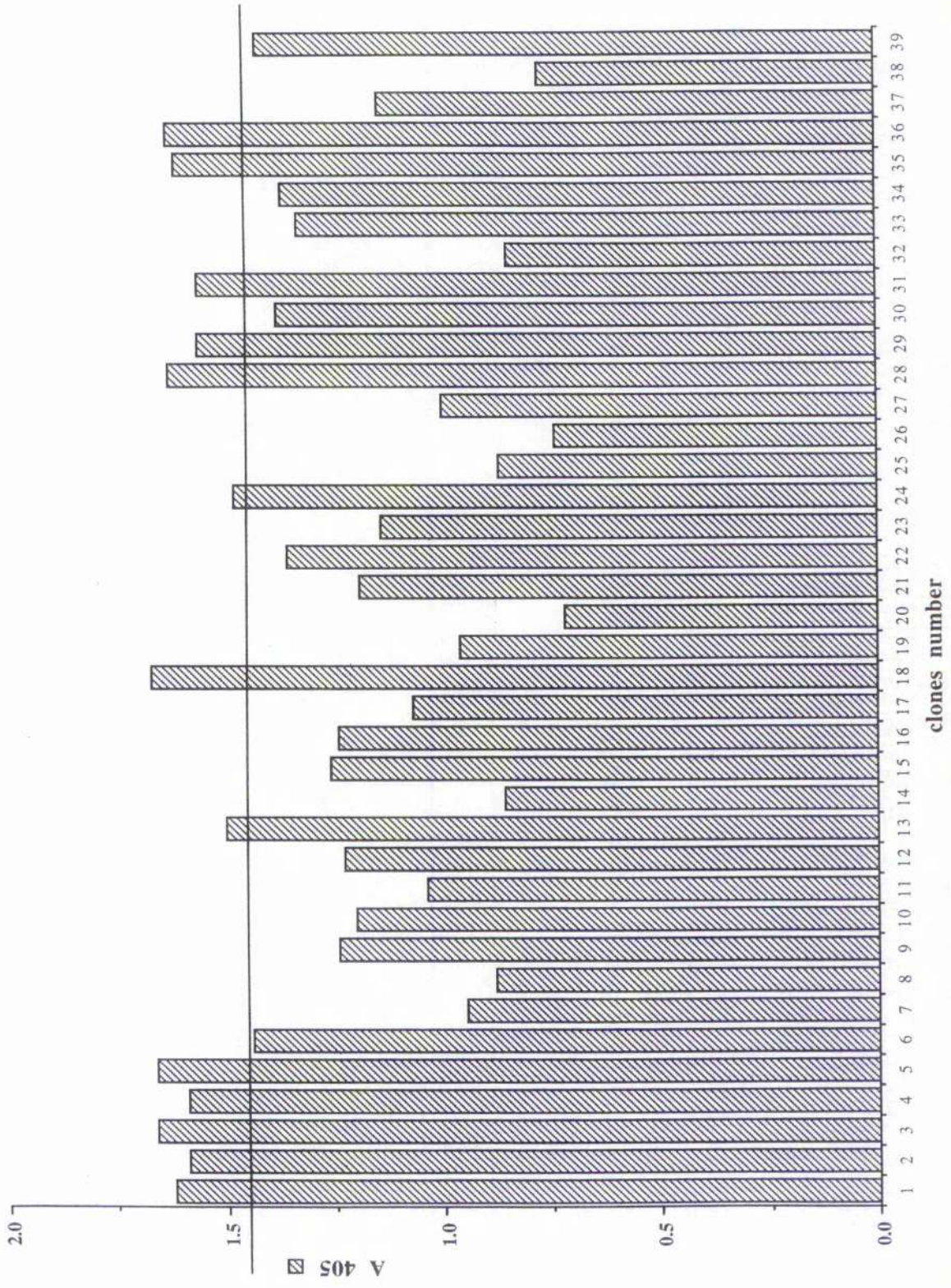

A 405
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
clones number

Figure 15. ELISA of 40 phage selected by biopanning with anti-CPS antiserum.

The microtitre plate was coated with purified phage (in duplicate wells) and subsequently treated with biotinylated rabbit anti-CPS antiserum, and biotinylated goat anti-rabbit IgG antiserum. Development was with HPR avidin and ABTS. The reaction was terminated after 8 minutes. Phage used were selected from the 6-mer library with biotinylated anti-CPS antiserum. The absorbency values for each phage have also been placed above the corresponding bars.

A 405
0.0
0.1
0.2
clone number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
0.164
0.193
0.172
0.124
0.199
0.08
0.079
0.079
0.092
0.078
0.077
0.078
0.129
0.091
0.093
0.0715
0.071
0.155
0.073
0.071
0.076
0.07
0.076
0.069
0.074
0.073
0.083
0.189
0.188
0.079
0.169
0.091
0.082
0.082
0.177
0.157
0.101
0.096
0.174
0.125

3.4.2 Reproducibility of ELISA using bound phage and anti-CPS antiserum

To determine the reproducibility of the ELISA method using anti-CPS antibody and microtitre-plate-bound phage, three putative high binding phage (5, 18 and 28) were assayed along with two low binding phage (25 and 26) using six wells per phage. The results shown in Figure 16 indicate marginal differences, demonstrating the consistency of the ELISA assay. This is confirmed in Table 3 where the mean and standard deviation of 6 replicate samples is given for each of the phage tested.

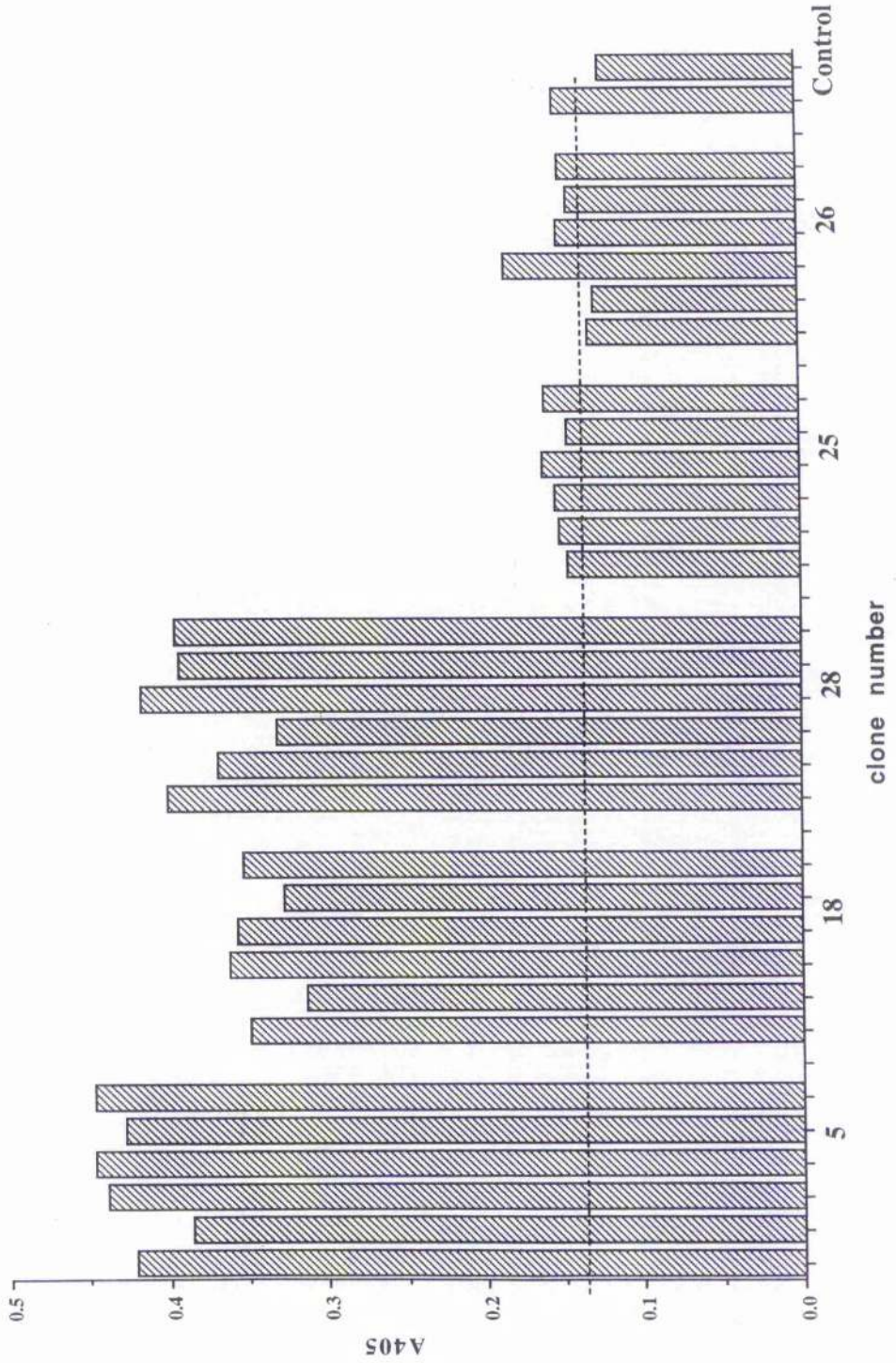
3.4.3 Effect of phage concentration on ELISA

To determine whether the amount of phage used for coating the ELISA plates might affect the subsequent A_{405nm}, phage from clones 1, 2 and 6, representing 2 putative 'high' and 1 putative 'low' binding affinity phage, respectively, were used to coat wells

Table 3. Mean and standard deviation for the ELISA absorbance values for phage 5, 8, 28, 25 and 26 selected by biopanning with anti-CPS antiserum.

Phage number	Absorbance (nm) mean (n = 6)	Standard deviation
5	0.430	0.023
18	0.343	.0019
28	0.385	0.030
25	0.153	0.007
26	0.150	0.020

Figure 16. Determination of tbe reproducibility of the ELISA for binding of phage by anti-CPS antiserum. The binding of phage 1, 18, 25, 26, and 28 was compared (6 wells per phage) and the reaction was terminated after 60 minutes.
The dotted line shows the mean absorbance value for the duplicate control wells containing TBS instead of phage.


A405
0.0
0.1
0.2
0.3
0.4
0.5
5
18
28
25
26
Control
clone number

of an ELISA plate at dilutions from neat to 1/100. Figure 17 shows the relationship between concentration of phage and ELISA absorbance. TBS buffer was used as a control and, as expected, the absorbance values for phage 6 at all dilutions were similar to those of the TBS control. For phage 1 the absorbance reading remained relatively constant up to a dilution of 1/10 and for phage 2 a dilution of 1 in 10 reduced the absorbance to background level (Figure 17).

3.4.4 Competitive ELISA

The competitive effect of purified CPS on the ability of the CPS antibody to bind to four phages which showed high binding in the ELISA (5, 2, 40, and 1; Figure 15) and two which showed low binding (6 and 26; figure 15) was investigated. Various concentrations of CPS were included with anti-CPS antiserum exposed to phage bound to the wells of the microtitre plate. Control wells gave the expected background OD at 405nm of approximately 0.1 (Figure 18). The high binding phage all gave absorbance values > 0.2 in the absence of CPS, and the low binding phage 26 gave a value similar to the background. However, an unexpected result occurred with phage 6, previously shown to be a low binding phage, which in this experiment gave an absorbance of 0.18 OD, a value similar to those of the high binding phage 2, 40 and 1. Since all the phage selected for this experiment had been re-amplified to produce sufficient material for the experiment, it is possible that phage 6 had not been properly amplified for previous experiments (Figure 15). Addition of CPS did not inhibit binding of antibody to the bound phage in a dose-dependent manner, although phage 5, 6, 2, and 40 showed maximum inhibition with CPS concentrations of 1 or 0.32 μg/ml (Figure 18). When CPS was bound to the microtitre plate for ELISA low absorbance values were found (far right of Figure 18), indicating either weak interaction between CPS and anti-CPS or poor binding of CPS to the plastic wells (see next section).

3.4.5 Binding of CPS to microtitre plates at different pH

The bicarbonate buffer normally employed for coating microtitre plate wells with antigen is designed for use with most protein antigens, but polysaccharide antigens

Figure 17. ELISA using various dilutions of phages of 1, 2 and 6 selected with biotinylated anti-CPS antiserum.

The TBS control (background level) is shown as a dotted line on the graph. The ELISA was terminated at 60 minutes. Phage 1, 2 and 6 were selected as representatives of all the phage isolated by biopanning.

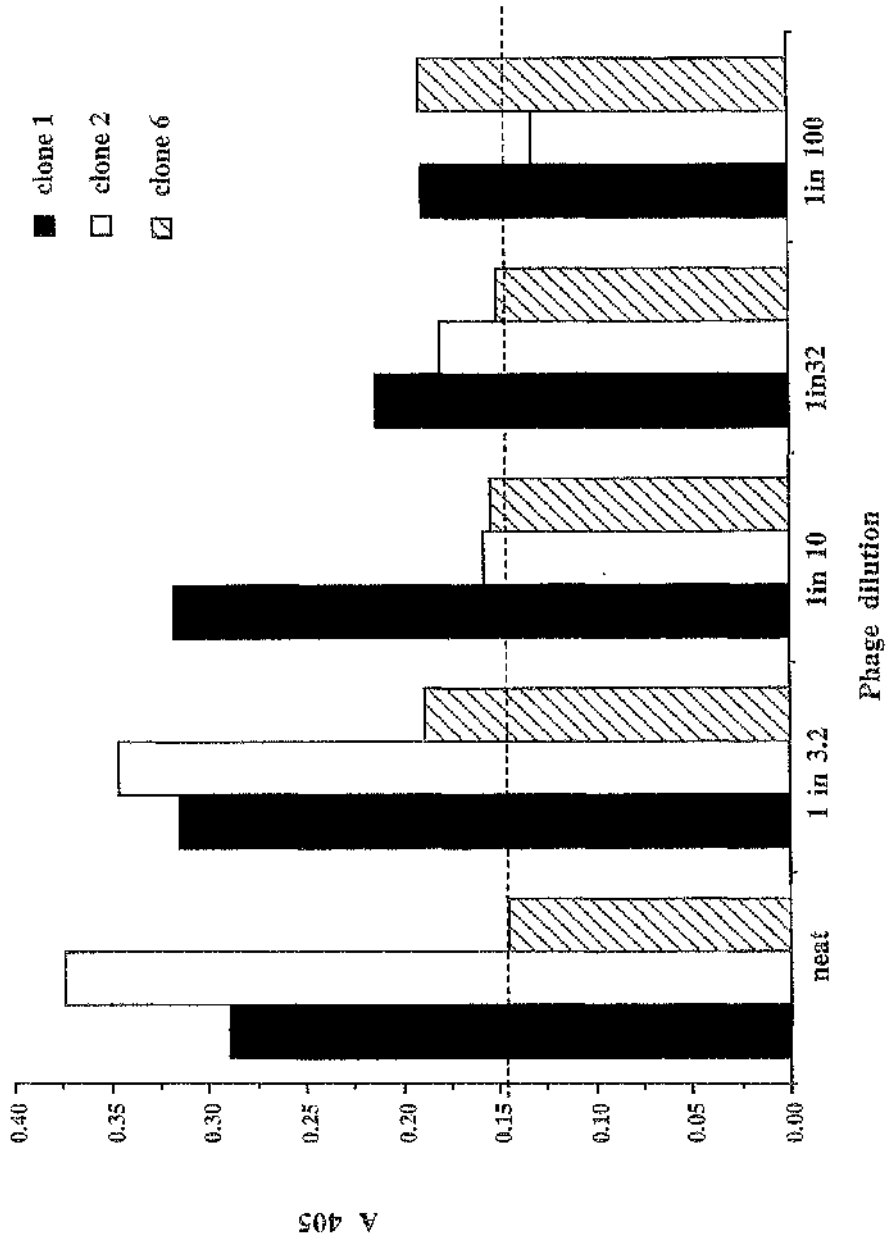

clone 1
clone 2
clone 6
neat
1 in 3.2
1in 10
1in32
1in 100
Phage dilution
A 405
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00

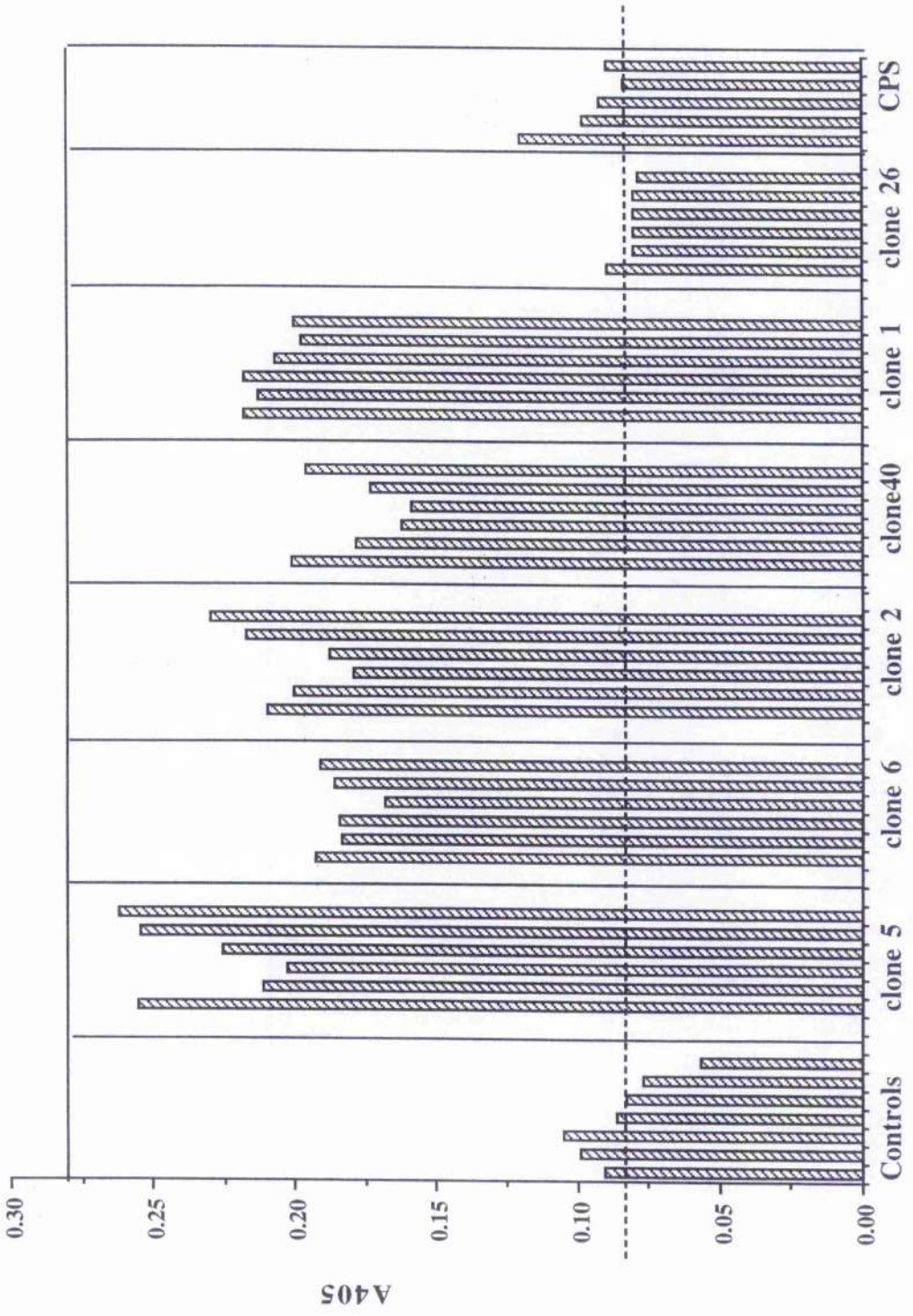
Figure 18. Effect of CPS on the binding of phage to anti-CPS antiserum.

Four phage (5, 2, 40 and 1) with putative high affinity binding to anti-CPS antisera and two (phage 6 and 26) of putative low affinity for anti-CPS antiserum were tested by ELISA for their ability to bind anti-CPS antiserum in the presence of 0-10 mg/ml CPS solution

Controls (from left to right) no phage, stock phage, (neat, 1/3, 1/10, 1/32), no antibody and no HRP. The average of these controls is indicated as a dotted line on the graph.

For clones 5, 6, 2, 40, 1 and 26. from left to right,
CPS was added to a concentration of 10 µg/ml, 3.2 µg/ml, 1 µg/ml, 0.32 µg/ml, 0,1 µg/ml, 0.0 µg/ml respectively.

CPS was used to coat certain wells of the microtitre plate instead of phage: concentrations used were (from left to right) 10 µg/ml, 3.2µg/ml, 1 µg/ml, 0.32 µg/ml, 0.1 µg/ml.


A405
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Controls
clone 5
clone 6
clone 2
clone40
clone 1
clone 26
CPS

often require different conditions for binding to occur. In this experiment the concentration of CPS was varied from 2.5 to 320 µg/ml and the buffer pH was varied from 5 to 9 to determine the effect on binding of CPS to ELISA plates. After deduction of background values it was apparent that absorbance values were still relatively low, but greatest binding occurred, on average, at pH 9 and was proportional to CPS concentration (Figure 19).

3.5 Determination of insert sequences

3.5.1 Phage selected after two rounds of biopanning with anti-CPS antiserum

Of the 40 phage analysed by ELISA (section 3.4), 17 were purified for sequence determination and the results are summarised in Figure 20. Representative examples of the sequencing lanes seen on the autoradiograph are shown in Figure 21 for phage 28, 29 and 36. From ELISA experiments 13 of the phage had been categorised as high binding clones and 4 as low binding.

It is interesting to note that 10 of the 17 phage have the initial insert sequence of 'GLY-SER-GLY', and that two such phage (14 and 20) were considered low binding clones. Clone 40 of the high binding group was found to have only a 6 nucleotide insert, clearly seen in Figure 22, and the 2 amino acid insert was 'GLY-SER', corresponding to the initial sequence found for the above group of 10 phage.

Figure 19. Effect of pH on binding of CPS to microtitre plates for ELISA.

The control to which no CPS was added, are shown separately. CPS at concentrations up to 320 μg/ml was used to coat the wells of a microtitre plate in buffers of pH 5 to pH 9, prior to reaction with biotinylated anti CPS antiserum and HRP-avidin.

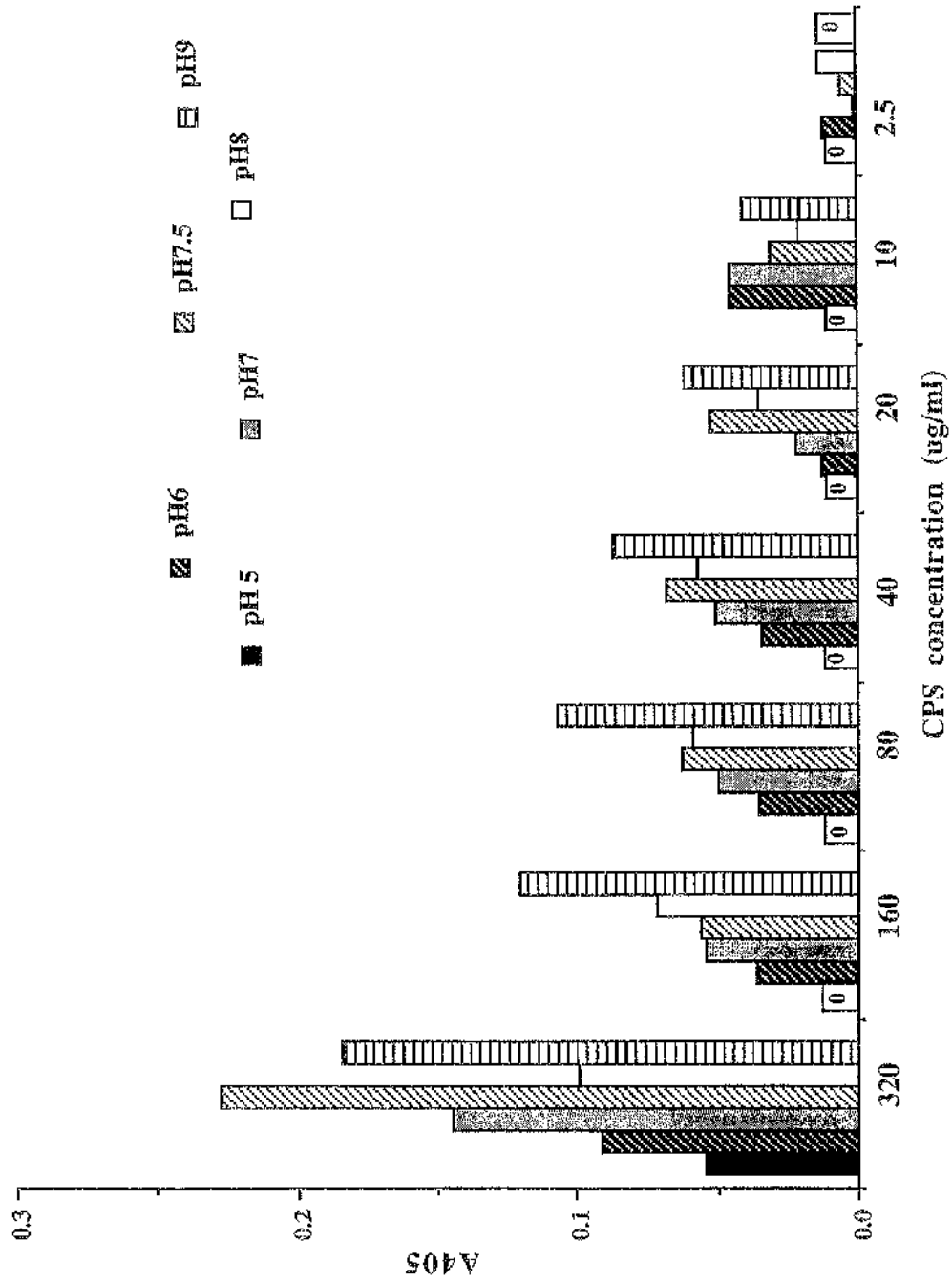

pH6
pH7.5
pH9
pH 5
pH7
pH8
A405
0.3
0.2
0.1
0.0
320
160
80
40
20
10
2.5
0
CPS concentration (ug/ml)

Figure 20. DNA and protein sequences of inserts from phage selected after two rounds of biopanning with CPS antiserum and the 6-mer phage library. Phage bound in the first round of biopanning were eluted with glycine/HCl buffer and from the second round with CPS (see Table 2). From the ELISA results (Figures 13 and 14) the phage were categorised into groups with high or low binding affinity. Note that an identical sequence of amino acids can arise from a different genetic code. The ? symbol indicates that it was difficult to read the nucleotide sequence from the autoradiograph.

HIGH AFFINITY BINDING PHAGE

Phage number	Amino acid sequence						genetic code
31	GLY	SER	GLY	ALA	HIS	TRP	(GGT TCT GGG GCT CAT TGG)
39	GLY	SER	GLY	ALA	HIS	TRP	(GGT TCT GGG GCT CAT TGG)
3	GLY	SER	GLY	ALA	ARG	LEU	(GGT TCT GGG GCT CGG CTT)
28	GLY	SER	GLY	GLY	MET	ASP	(GGT TCT GGT GGT ATG GAT)
13	GLY	SER	GLY	ARG	ASP	ALA	(GGT TCT GGG CGT AAT GCT)
4	GLY	SER	GLY	SER	ARG	THR	(GGT TCT GGG AGT CGT ACG)
2	GLY	SER	GLY	PRO	VAL	ASN	(GGG TCT GGT CCG GTT AAT)
5	GLY	SER	GLY	PRO	ALA	VAL	(GGG TCT GGG CCG GCG GTT)
1	GLY	THR	GLY	SER	TRP	GLY	(GGT ACT GGG TCT TGG GGT)
36	MET	PHE	SER	LEU	ILE	PRO	(ATG TTT TCG CTT ATT CCT)
18	PRO	TYR	SER	PRO	HIS	LEU	(CCG TAT AGT CCT CAT CTG)
29	SER	GLN	ALA	ARG	SER	GLY	(TCG CAG GCT CGT TCT GGT)?
40	GLY	SER					(GGT AGT) (2aa INSERT)

LOW AFFINITY BINDING PHAGE

14	GLY	SER	GLY	GLY	GLU	HIS	(GGT TCG GGG GGT GAG CAT)
20	GLY	SER	GLY	GLY	GLU	HIS	(GGT TCG GGG GGT GAG CAT)
8	SER	SER	MET	LEU	PRO	PRO	(TCG TCG ATG CTG CCT CCT)
32	SER	GLN	ASN	LEU	ASN	GLU	(TCG CAG AAT CTT AAT GAG)?

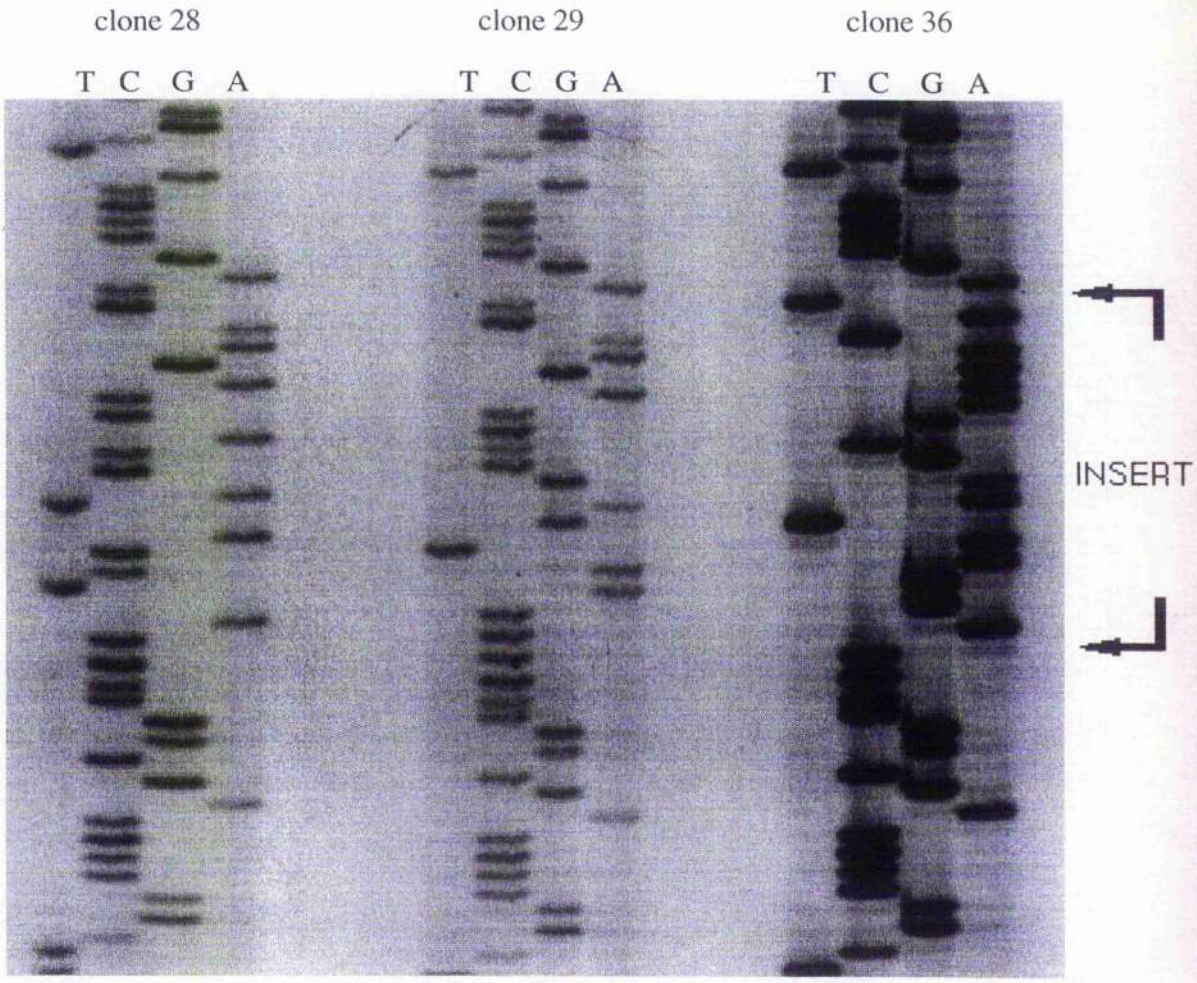


FIGURE 21. Example of an autoradiograph for nucleotide sequence determination.

DNA extracted from phage was used for nucleotide sequence determination. The results for phage 28, 29, and 36 selected from the 6-mer library by biopaning with biotinylated anti-CPS antiserum are shown. The insert is located between the arrows as shown.

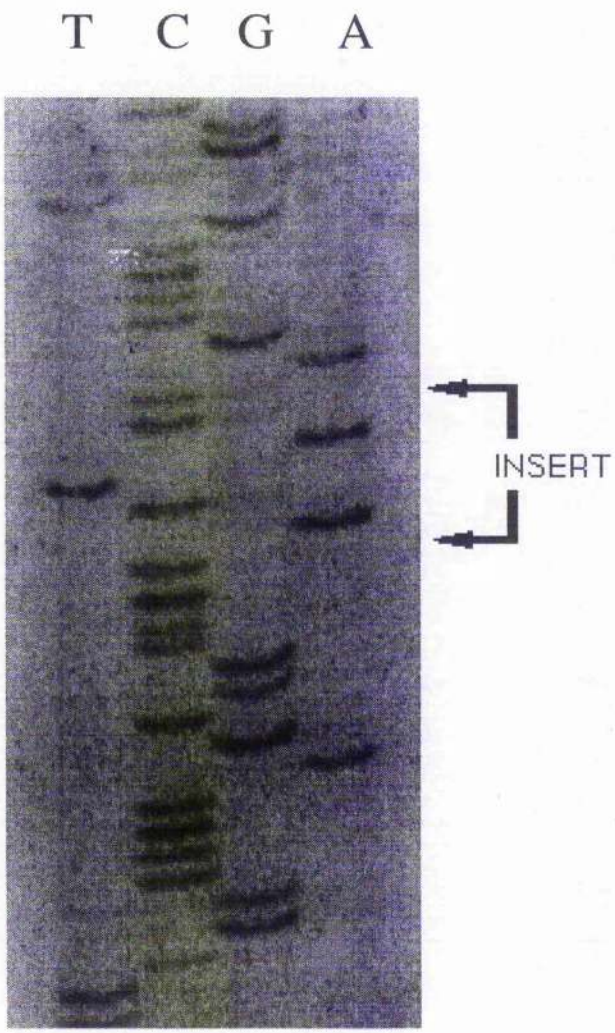


FIGURE 22. Autoradiograph showing the 6 nucleotide insert sequence of phage 40, selected with anti-CPS antiserum

Phage 40 was selected from the 6-mer phage library by biopanning with anti-CPS antiserum, being eluted by glycine/HCl in the first round of biopanning and CPS in the second round. The six base insert (coding for 2 amino acids) is located directly above the middle group of 4 cytosines. All other phage selected from this round contained a DNA insert coding for six amino acids.

3.6 Repeat amplification of second round eluate and further biopanning using anti-CPS antiserum and CPS as the elutant, to produce a third round eluate.

To further refine the selection of phage from the two rounds previously described the phage recovered in the second round were re-amplified and taken through a third round of biopanning with the biotinylated anti-CPS antiserum and CPS solution as the elutant. The titre of phage used in round three (Table 2.) was calculated to be 1.3×10^{15} virions/ml with an actual input of 1.27×10^{14} virions for the 98 µl added. The titre of the recovered phage (Table 2) was 2.9×10^{7} and 1.9×10^{7} virions/ml for tubes A and B, respectively, with a total yield of 1.4×10^{7} and 9×10^{6} virions, respectively. The percentage yields for the third round of biopanning were 1.1×10^{-5} and 6.9×10^{-6} % for tubes A and B. These low values may be due to the reduced amount of antibody used in this round in order to promote competition and selection of phage with the highest binding affinity. It may also be that the titre of the input phage was erroneously high.

Eighty phage obtained from the above biopanning experiment were selected for further analysis (Table 2).

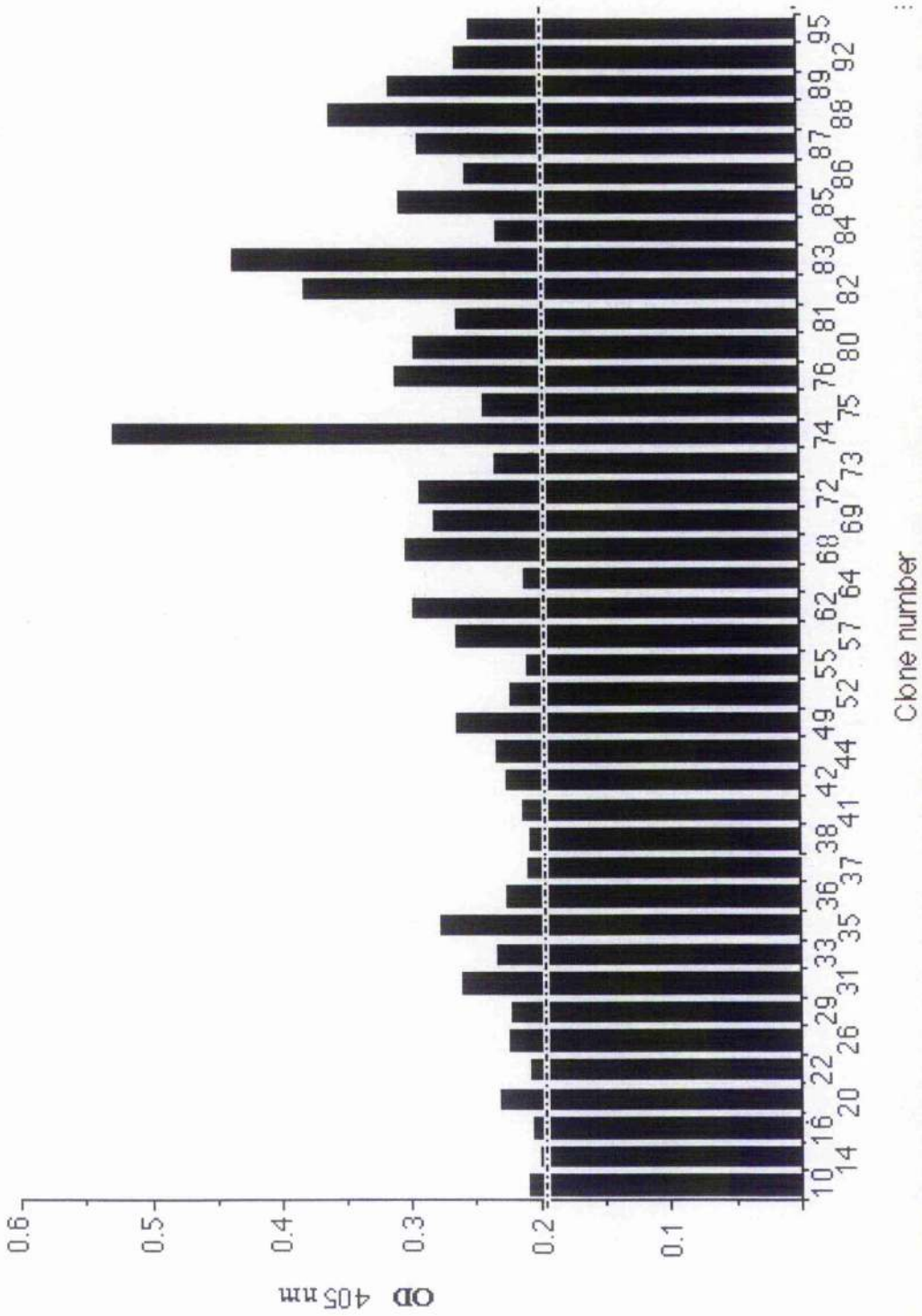
3.6.1 ELISA of phage selected in a third round of biopanning with anti-CPS antiserum and eluted with CPS

Phage from all 80 clones were bound to microtitre plates and tested by ELISA for their binding of biotinylated rabbit anti-CPS antiserum. Figure 23 shows the results for those phage which yielded absorbance values above the level of the background control, clone 78, a phage shown by sequencing to contain no insert and, therefore, one which should show no specific binding to the anti-CPS antibodies.

3.6.2 Estimation of concentration of phage by ELISA

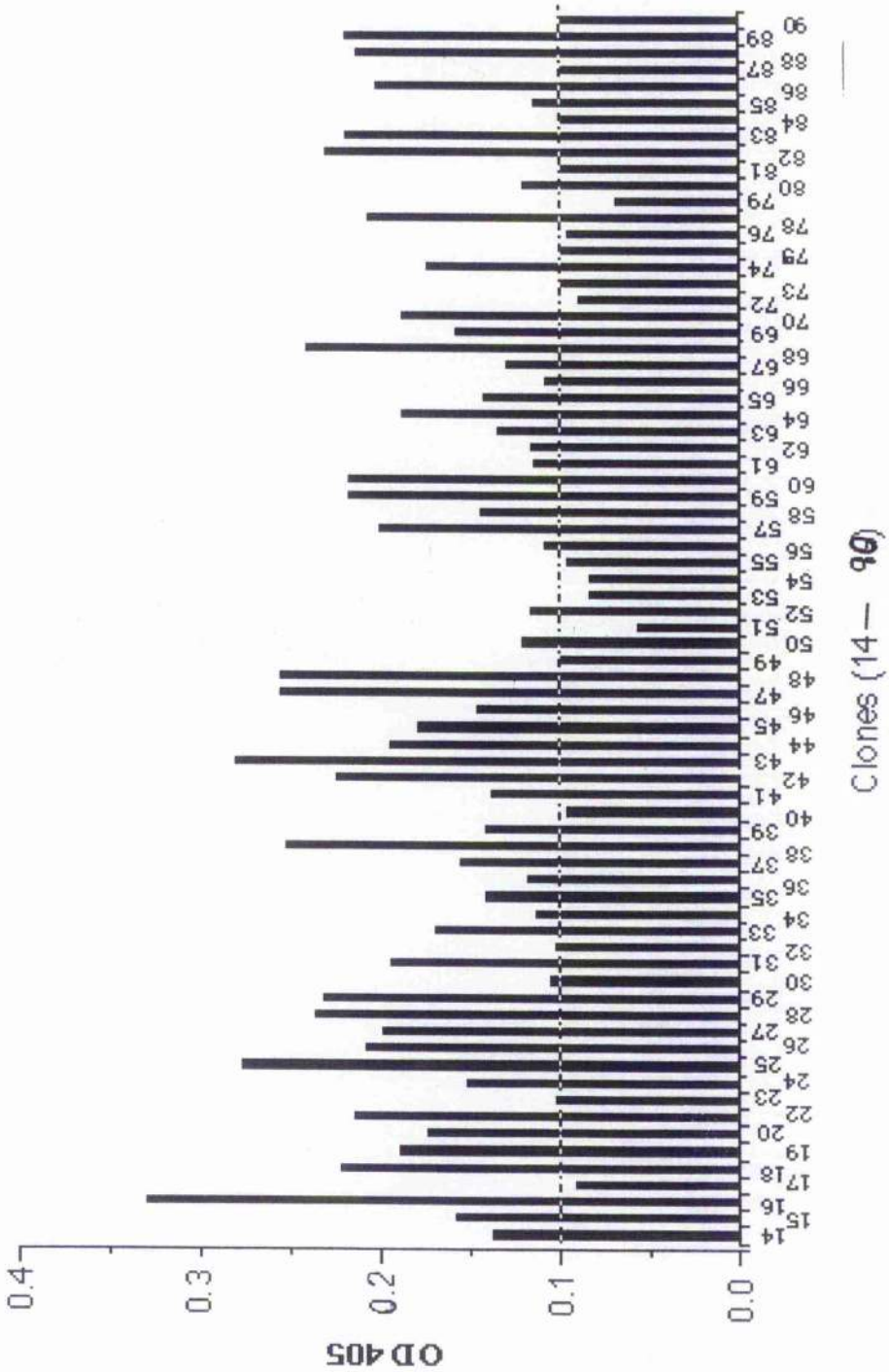
Determination of the concentration of fd-tet phage in a suspension is difficult (Smith, 1993) as the virus does not form clear plaques, and transformation of *E. coli* to yield tetracycline-resistant transformants occurs with variable, usually low, efficiency. Therefore, quantification by ELISA, using an HRP-labelled anti-M13 antiserum, was

Figure 23. ELISA of 80 isolated phage selected by biopanning a 6-mer library with anti-CPS antiserum. The 1st round elution was with glycine/HCl and the 2nd and 3rd round with CPS (120 μg/400 ml). The background reading is shown by the dotted line. Only those phage with results higher than background are shown.


OD 405nm
0.6
0.5
0.4
0.3
0.2
0.1
10 14 16 20 22 26 29 31 33 35 36 37 38 41 42 44 49 52 55 57 62 64 68 69 72 73 74 75 76 80 81 82 83 84 85 86 87 88 89 92 95
Clone number

used to determine whether certain phage had been amplified adequately for the anti-CPS-binding ELISA. The results (Figure 24) show that the concentration of the purified phage varied widely with 20 of the phage tested giving absorbance values close to the background control level. Such phage probably require re-amplification to a higher concentration before reliance can be place on ELISA results to assess binding to anti-CPS antibodies. However an absorbance value significantly above background (Figures 14 and 15) could be taken as evidence that that particular phage was capable of binding to anti-CPS antibody. A few of the phage whch gave positive results (shown in Figure 24) were tested at various dilutions for comparison with the 1×10^{13} virions/ml stock phage library (Figure 25).

Figure 24 . ELISA for detection of fd phage using anti-M13 antiserum. ELISA of 74 phage, selected by biopanning with biotinylated anti-CPS. Of the 96 phage obtained in biopanning with anti-CPS MAb, 74 were used to coat microtitre plate wells in an ELISA in which phage were detected with anti rabbit anti-phage-M13 antiserum and HRP-labelled goat anti-rabbit-IgG antiserum. (phage 21, 71, & 77 have been omitted)


OD 405
0.0
0.1
0.2
0.3
0.4
Clones (14 – 90)

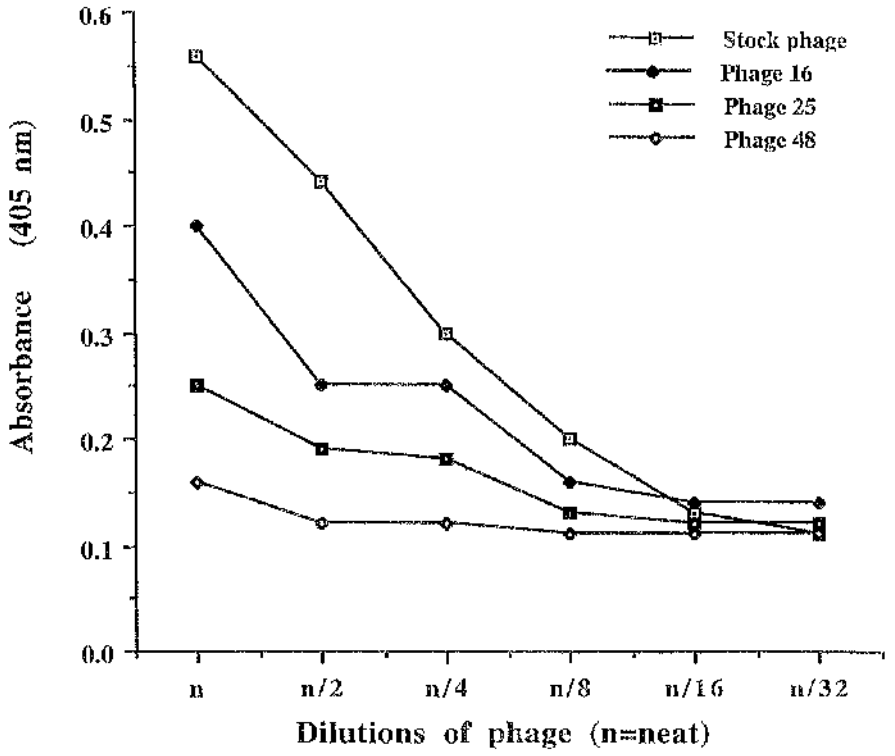


Figure 25. Effect of phage concentration in the phage anti-M13 antibody ELISA

The phage tested were numbers 16, 25, and 48 selected by biopanning with biotinylated anti-CPS antiserum, and the 6-mer phage library stock (1×10^{15} virions/ml)

3.7 Determination of DNA sequences of inserts in phage eluted with CPS in a third round of biopanning with anti-CPS antiserum

In Figure 26 the DNA sequences of inserts in 22 phage chosen for analysis are shown with the amino acid sequences encoded by the inserts. Three groups of identical sequences were found, each containing two members. The other sequences appear to have little in common, and there was no obvious relationship with the sequences determined for the earlier round of elution with CPS (Figure 20). Two sequences of particular interest were phage 15, containing a five amino acid insert, and phage 78, which contained no insert. The sequences of four clones were unreadable probably because the concentration of DNA was too low for sequencing.

Figure 26. Amino acid sequences of inserts in proteins of phage selected from the 6-mer library in a third round of biopanning with anti-CPS antiserum

In the first round of biopanning phage were eluted with glycine/HCl and in the second and third rounds with CPS (See Figure 9).

Phage number	Amino acid sequence	genetic code
50 -	TRP ARG ASN TRP ARG HIS	(TGG AGG AAT TGG CGT CAT)
71 -	TRP ARG ASN TRP ARG HIS	(TGG AGG AAT TGG CGT CAT)
85 -	TYR HIS PHE ALA ARG THR	(TAT CAT TTT GCT CGT ACT)
90 -	TYR HIS PHE ALA ARG THR	(TAT CAT TTT GCT CGT ACT)
20 -	PRO ARG LEU PRO PHE SER	(CCT CGG CTG CCT TTT TCG)
37 -	PRO ARG LEU PRO PHE SER	(CCT CGG CTG CCT TTT TCG)
54 -	PHE TYR SER ALA SER ISO	(TTT TAT TCG GCG TCT ATT)
59 -	TYR HIS LEU SER LEU ASN	(TAT CAT TTG AGT CTG AAT)
15 -	PRO SER ILE SER GLN (5-MER)	(CCG TCT ATT TCG CAG)
88 -	PRO GLY LEU PHE HIS TYR	(CCG GGT CTG TTT CAT TAT)
10 -	LYS PRO SER ARG THR VAL	(AAG CCT AGT CGG ACG GTT)
67 -	LYS PHE ALA LYS VAL ARG	(AAG TTT GCT AAG GTG AGG)
4 -	MET LYS GLY GLU ALA ASN	(ATG AAG GGG GAG GCT AAT)
58 -	MET MET SER LEU THR ARG	(ATG ATG AGT TTG ACG CGT)
25 -	ILE GLY TYR LEU GLY GLY	(ATT GGT TAT CTT GGT CGG)
81 -	VAL GLY ARG SER VAL LEU	(GTT GGG CGG TCG GTT CTT)
87 -	ALA GLY ARG TYR LEU HIS	(GCT GGG CGG TAT CTT CAT)

78 -WT (wild type, no insert)

1X 30X 35X 40X (unreadable)

3.8 Biopanning of the Smith 6-mer phage library using monoclonal antibody to *A. salmonicida* LPS

One of the difficulties inherent in using a polyvalent antiserum is the variety of epitopes to which antibodies will be present and the difference in affinity of different antibodies directed against the same epitope. This can be eliminated by using monoclonal antibodies (MAb) as they are homogenous and it can be assumed that they bind to a single epitope.

3.8.1 Biotinylation of anti-LPS monoclonal antibody

The anti-LPS MAb 'F9 clone 16'was biotinylated and separated from unreacted biotin by gel filtration (Figure 27). Fractions 4 and 5 were pooled and contained 1.1 mg/ml protein, with a biotin/protein ratio of 6.

3.8.2 Biopanning with biotinylated anti LPS monoclonal antibody

Three rounds of biopanning were executed as described in the Materials and Methods (section 2.4.11).

3.8.3 ELISA of phage selected by biopanning with anti-LPS MAb

Phages recovered from the three rounds of biopanning with anti-LPS MAb were amplified and purified for ELISA, using method C (Section 2.6.4). The results are shown in Figure 28. The high background absorbance at 405nm of the control, almost 0.6, is shown by the dotted line. Each of the three ELISA plates used for this experiment contained duplicate samples of LPS at three concentrations, the lowest of which, 0.1 µg/ml LPS, resulted in a very low absorbance, although concentrations of 10 and 33 µg/ml LPS gave readily detectable responses. The results for the phage were all below that of the control value and the average values for groups of different origin are shown (see Figure 28), to simplify presentation. It would appear that no high affinity binding phage had been selected in the biopanning with anti- LPS antibody. Attempts to improve the ELISA were unsuccessful and it was decided to determine the

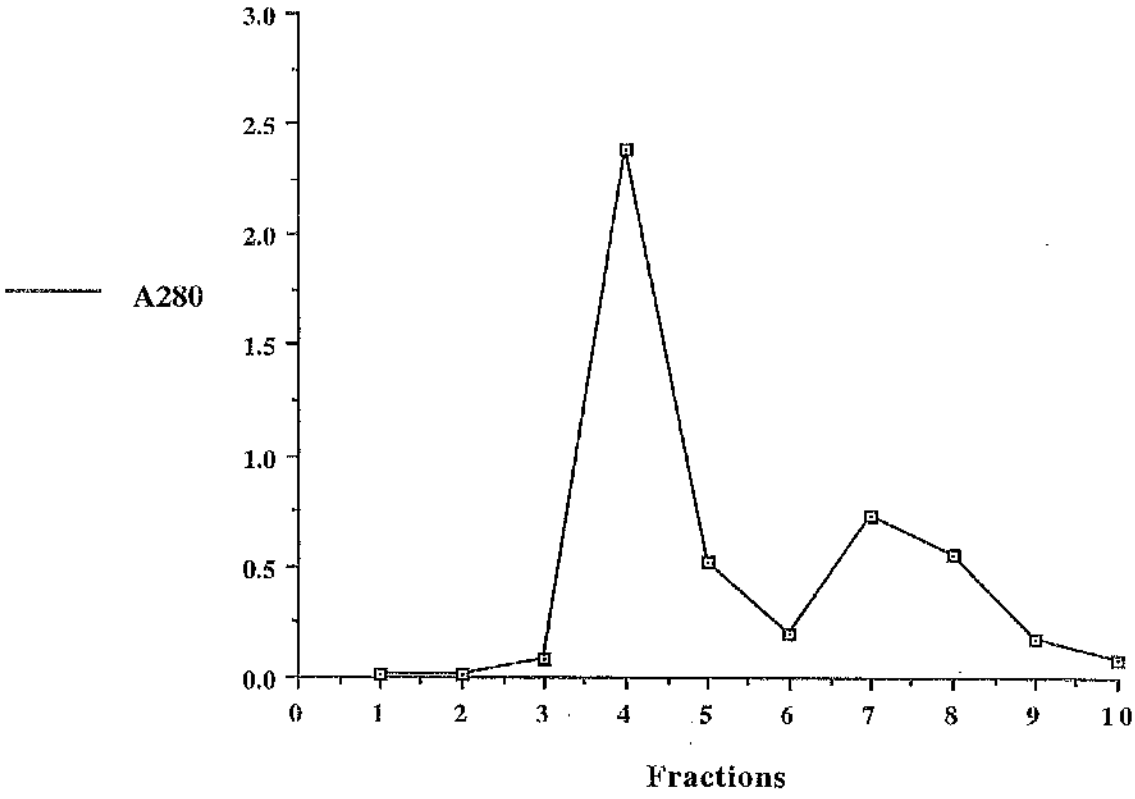


Figure 27. Separation of biotinylated anti-LPS immunoglobulin from free biotin by Sephadex G 25 chromatography.

One ml biotinylated immunoglobulin reaction mixture from rabbit antiserum to *A. salmonicida* CPS was applied to a column of Sephadex G25 Fractions of 1 ml were collected and the absorbance at 280nm measured. Fractions 4 and 5 were combined to give 4 ml of biotinylated antibody. Free biotin was eluted in subsequent fractions (not shown).

Figure 28. ELISA of 96 phage selected by biopanning with anti-LPS monoclonal antibody

This ELISA was carried out on three plates which each contained a set of LPS controls in which LPS was added instead of phage, in duplicate, at concentration of 0.1, 10 and 33 mg/ml.
TBS (instead of phage) control (background level) is indicated as a dotted line on the graph.

The phage results were averaged in group order as follows.

1st round

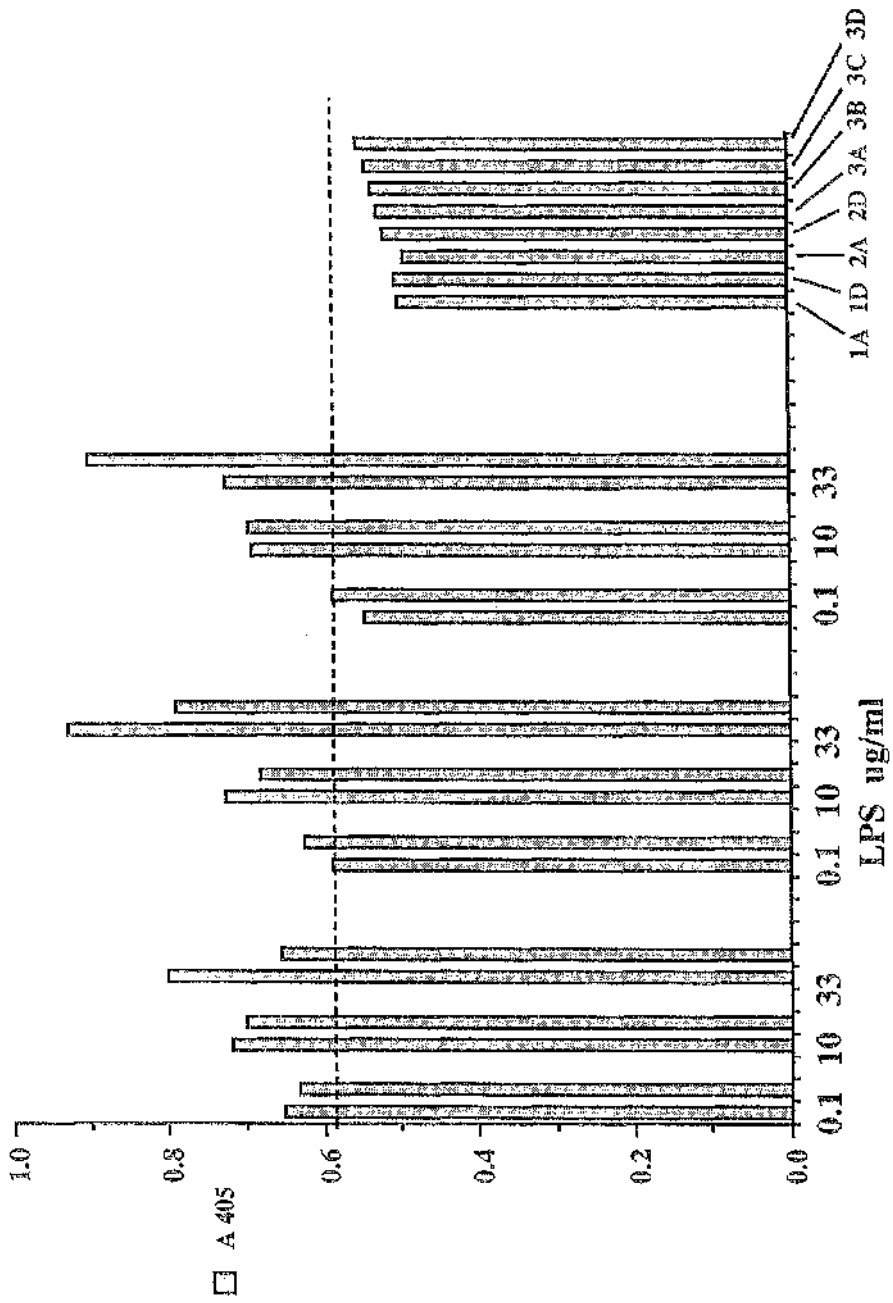
1A	=	Tube 1 / 1st wash	(clones 2-6)
1D	=	Tube 2 / 2nd wash	(clones 7-10)

2nd round

2A	=	Tube 1 / 1st wash	(clones 11-15)
2D	=	Tube 2 / 2nd wash	(clones 16-20)

3rd round

3A	=	Tube 1 / 1st wash	(clones 21-51)
3B	=	Tube 1 / 2nd wash	(clones 52-65)
3C	=	Tube 2 / 1st wash	(clones 66-76)
3D	=	Tube 2 / 2nd wash	(clones 77-96)


A 405
1.0
0.8
0.6
0.4
0.2
0.0
0.1 10 33
0.1 10 33
0.1 10 33
LPS ug/ml
1A 1D 2A 2D 3A 3B 3C 3D

insert sequences of the phage to look for any bias of selection which might be detected by sequence similarities.

3.8.4 DNA sequence determination for 6-mer phages selected by biopanning with anti-LPS monoclonal antibody

The peptide inserts coded for by these phage are shown in Figure 29; 5 main sequence groups were found and the numbers of these phage have been listed along with their group sequence to simplify presentation. Against each sequence is shown a code to indicate its origin e.g A3 (see methods).

The genetic codes for the amino acid inserts are shown since identical amino acid inserts could arise from different DNA sequences It is of interest to note that identical phage had been selected in the parallel biopaning experiments in tubes A to D, e.g. phage 70 from C3 is identical to phage from A3. This is also shown for phage 80 from D3 and phage 66 from C3, however, these clones did originate from the same product of the first round of biopanning. Phage 57 contained the wild type gene iii (see figure 30) and phage 18 contained a 5 amino acid insert; the significance of which is discussed later.

Phage 80 which has the sequence ASN LEU MET ARG LEU TYR, can be compared to Phage 82 which has the sequence ILE SER ARG MET LEU PHE. The 3 amino acids underlined are reversed in the two clones as are the (TRP ARG HIS) sequences found in phage 47, reversed in phage 68.

Certain phage isolated during LPS biopanning had identical insert sequences to phage isolated during biopanning with anti-CPS antiserum, e.g. phage 18 from the second round of CPS biopanning has a sequence identical to that from anti-LPS biopanning (phage 79). From the 3rd round of anti-CPS biopanning phage 50, 90, 54, 15, 4, 81 and 87 are identical to, respectively, LPS clones 21, 75, 53, 71, 13, 42 and 65.

The significance of this is discussed in more detail later.

Figure 29. Insert sequences of phage isolated by three rounds of biopanning with the 6-mer phage library and biotinylated anti-LPS monoclonal antibody.

In three rounds, bound phage were eluted with glcine/HCl buffer. See figure 10 for the origin of the individual phage and the method of isolation.

Insert Amino acid sequence	DNA sequence	Origin	Phage with identical sequences (origin)		Phage with related sequences (similarity)
ASN LEU MET ARG LEU TYR	(AAT CTT ATG AGG TTG TAT)	37 A3	52, 54, 62, 63, & 64 66, 67, 69, & 72 80	B3 C3 D3	10, 93, 77, 78 (LEU TYR)
TRP ARG ASN TRP ARG HIS	(TGG AGG AAT TGG CGT CAT)	21 A3	25, 29, 33, 34, 39, 47 & 48 70	 A3 C3	68,58 (TRP ARG HIS GLY) In 21 GLY is next to HIS
PRO TYR SER PRO HIS LEU	(CCG TAT AGT CCT CAT CTG)	79 D3	81 85, 86,90, 91, 94 & 95	 D3	5, 17, 71(PRO SER)
ILE SER ARG MET LEU PHE	(ATT TCG CGG ATG CTG TTT)	74 C3	82, 83, 84, 96 & 87 88	 C3 D3	53, 61 In reverse, (ILE SER x x x PHE) 71 (ILE SER)
PHE TYR SER ALA SER ILE	(TTT TAT TCG GCG TCT ATT)	53 B3	61	B3	
LEU HIS ARG TRP GLY ARG	(TTG CAT AGG TGG GGT AGG)	68 C3	58	B3	73 (TRP ARG)
HIS VAL ALA ILE HIS SER	(CAT GTG GCG ATT CAT TCG)	22 A3	32	A3	
MET MET LEU PRO LEU TYR	(ATG ATG TTG CCG CTT TAT)	78 D3	77	D3	93 (PRO LEU TYR)

Figure 29. (CONTINUED). Insert sequences of phage isolated by three rounds of biopanning with the 6-mer phage library and biotinylated anti-LPS monoclonal antibody.

In three rounds, bound phage were eluted with glycine/HCl buffer. See figure 10 for the origin of the individual phage and the method of isolation.

Insert Amino acid sequence	DNA sequence	Origin		Phage with identical sequences (origin)	Phage with related sequences (origin)
CYS CYS LEU TYR ALA CYS	(TGT TGT TTG TAT GCG TGT)	10	D1		
MET ARG VAL SER THR GLN	(ATG CGG GTT AGT ACG CAG)	76	C3		
ASP MET LYS PRO LEU TYR	(GAT ATG AAG CCG CTT TAT)?	93	D3		
PHE ARG ASN LEU VAL TYR	(TTT CGT AAT TTG GTT TAT)	60	B3		
THR SER VAL HIS GLY SER	(ACT TCG GTG CAT GGT TCT)	41	A3		
VAL GLY ARG SER VAL LEU	(GTT GGG CGG TCG GTT CTT)	42	A3		
SER ARG SER ALA PRO MET	(TCG CGT TCT GCG CTT CAG)	26	A3		
ASP LYS TYR ALA LEU GLN	(GAT AAG TAT GCG CTT CAG)	27	A3		
LEU PHE ALA THR ALA GLY	(CTG TTT GCG ACT GCG GGG)	30	A3		
CYS PHE TRP VAL HIS CYS	(TGT TTT TGG GTG CAT TGT)?	59	B3		
GLY ASP MET HIS GLY GLY	(GGT GAT ATG CAT GGT GGT)	55	B3		
ALA GLY ARG HIS LEU HIS	(GCT GGG CGG CAT CTT CAT)?	65	B3		
THR VAL ILE ARG SER PHE	(ACT GTT ATT CGT TCG TTT)	56	B3		
GLU VAL SER LEU ARG TRP	(GAG GTG TCG CTT AGG TGG)	73	C3		
TYR HIS PHE ALA ARG THR	(TAT CAT TTT GCT CGT ACT)	75	C3		
PRO SER ILE SER GLN(5-MER)	(CCG TCT ATT TCG CAG)	71	C3		
GLY TRP SER PRO SER SER	(GGG TGG AGT CCG TCT AGT)	5	A1		
VAL TYR ALA PRO PRO PRO	(GTT TAT GCG CCG CCG CCG)?	4	A1		
SER LEU ARG ASN THR MET	(TCT CTG CGT AAT ACG ATG)?	12	A2		

Figure 29. (CONTINUED). Insert sequences of phage isolated by three rounds of biopanning with the 6-mer phage library and biotinylated anti-LPS monoclonal antibody.

In three rounds, bound phage were eluted with glycine/HCl buffer. See figure 10 for the origin of the individual phage and the method of isolation.

Insert Amino acid sequence	DNA sequence	Origin	Phage with identical sequences (origin)	Phage with related sequences (origin)
MET LYS GLY GLU ALA ASN	(ATG AAG GGG GAG GCT AAT)	13	A2	
ARG TRP THR ALA PHE ASP	(CGT TGG ACT GCG TTT GAT)?	14	A2	
PRO VAL SER TRP ILE TYR	(CCG GTT TCG TGG ATT TAT)	9	D1	
ARG LEU GLY GLN VAL TYR	(AGG CTG GGT CAG GTG TAT)	16	D2	
GLY ASN LEU SER PRO GLU	(GGT AAT CTT TCG CCG GAG)?	17	D2	
PHE LEU VAL CYS PRO PHE	(TTT TTG GTT TGT CCT TTT)?	20	D2	
GLU ALA ALA ILE ARG (5-MER)	(GAC GCT GCT ATT CGT)	18	D2	

unreadable sequences

19 X_{D2} 23X_{A3} 24X_{A3} 50?$_{A3}$ 51?$_{A3}$ 45.X_{A3} 89X_{D3}

57 WT_{B3}

31 ? ? ? ? VAL PHE_{A3} (? ? ? ? GTG TTT)

44 ? ? ? ? ? PHE_{A3} (? ? ? ? ? TTT)

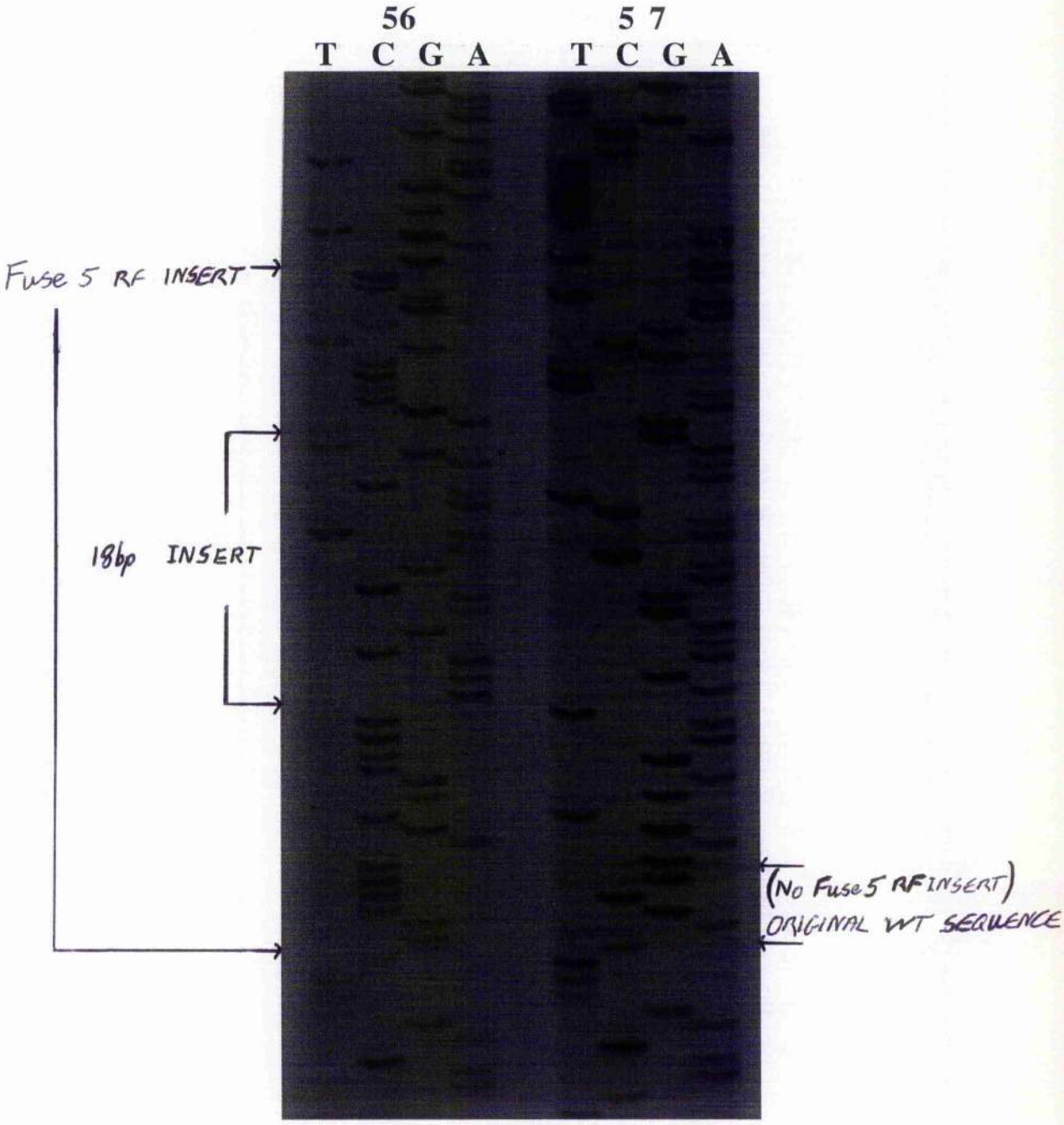


Figure 30. Autoradiograph showing the DNA sequences of phage 56 and 57. Phage 56 displays a 6 amino acid insert whereas phage 57 contains no insert. Both phage were selected from the last of three rounds of biopanning the 6-mer phage and anti-LPS MAb.

3.9 Biopanning of a 15-mer phage-displayed peptide library with biotinylated anti-LPS monoclonal antibody

The 15-mer phage library was treated in the same as the 6-mer library and three rounds of biopanning were completed (see methods).

3.9.1 ELISA of 15-mer phage selected using anti LPS monoclonal antibody

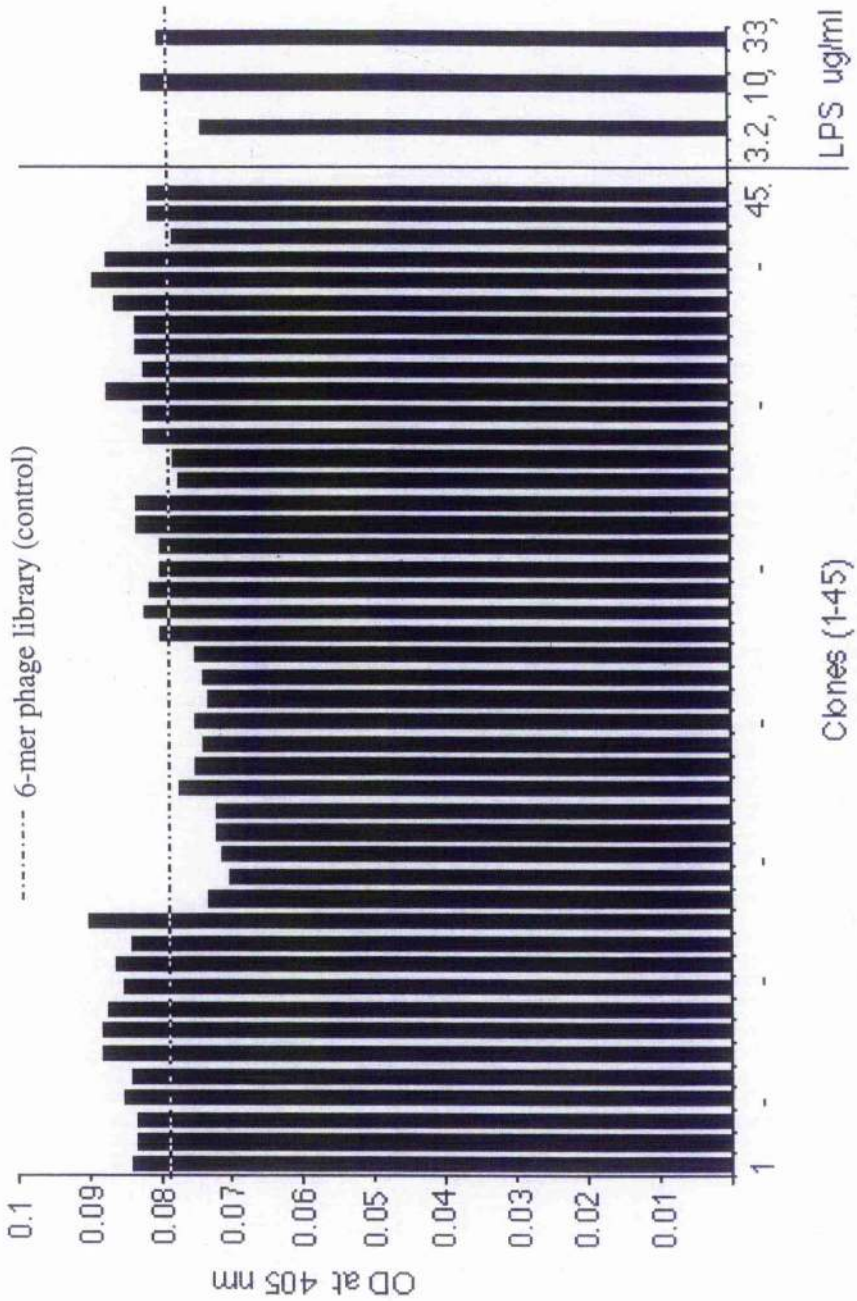
The ELISA results for the 45 phage are shown in Figure 31 with the background absorbance obtained using stock library phage shown by the dotted line. The absorbance values were similar to that of the control line. The LPS controls gave absorbance values only marginally above background which may have been due to poor binding of the LPS to the plate.

3.9.2 DNA sequences of the inserts in phage from the 15-mer library selected using biotinylated anti-LPS monoclonal antibody

Surprisingly, none of the sequences determined revealed 15 amino acid inserts (figure 32), there being 2 phage with six amino acid inserts and three with wild type gene III sequence (no insert).

Figure 31 . ELISA of 45 phage selected by biopanning a 15-mer library with anti-LPS monoclonal antibody

The microtitre plate was coated with purified phage (in duplicate wells) and subsequently treated with biotinylated anti-LPS monoclonal antibody. Development was with HPR avidin and ABTS. Phage used were selected from the 15-mer library with biotinylated anti-LPS monoclonal antibody.


OD at 405 nm
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
6-mer phage library (control)
1
45
Clones (1-45)
3.2, 10, 33
LPS ug/ml

Clone number	Amino acid sequence					genetic code
37	HIS	VAL	ALA	ILE	HIS	SER (CAT CTG GCG ATT CAT TCG)
40	HIS	VAL	ALA	ILE	HIS	SER (CAT CTG GCG ATT CAT TCG)
36	WT	(wild type)				
38	WT					
39	WT					

Figure 32. Amino acid sequences of inserts in phage selected by biopanning a 15-mer phage library with biotinylated anti-LPS monoclonal antibody

The 5 phage sequences were derived from phage recovered from tube 12 (see figure 11). The first round phage were recovered by elution of bound phage with glycine/HCl buffer and then by LPS in the subsequent two rounds.(see Figure 11 for full description).

3.10 Electron Microscopy of uninfected and phage-infected cells

Because wild-type phage sequences were found on a small number of occasions (Figures 26, 29 and 32) it was decided to investigate whether wild-type phage might be present in stocks of uninfected *E. coli* K91kan cells, and also to determine whether it might be feasible to obtain direct counts of phage preparations. When uninfected cells were viewed with the electron microscope no bacteriophage could be seen, compared with infected *E. coli* K91kan cells which contained readily visible numbers of phage (Figure 33).

Figure 33 shows an electron micrograph of two isolated phage lying parallel to each other. Although the staining was slightly uneven the entire length of the phage can be seen.

Figure 34 shows a single filamentous bacteriophage associated with an *E. coli* cell. It is possible that the proximal end of the phage is attached to the underside of the cell. It was not considered feasible to obtain total virion counts on a regular basis for large numbers of phage solutions generated in biopanning.

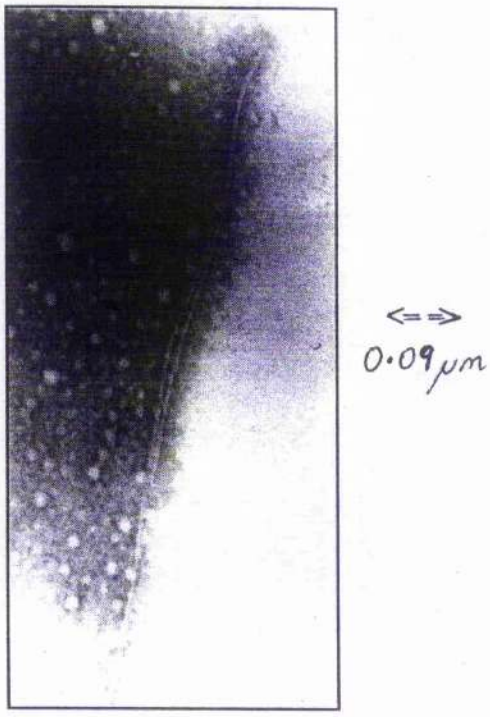


Figure 33. Filamentous bacteriophage viewed by Transmission electron microscopy photograph.

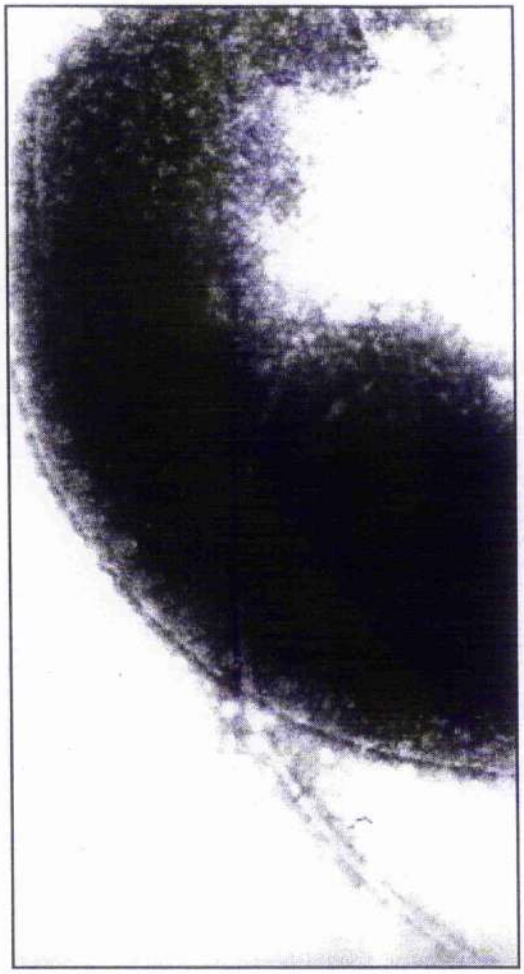


Figure 34. Transmission electron microsgraph of a phage infected *E. coli* cell.

4. DISCUSSION

Successful vaccines have now been produced commercially against several fish pathogens, such as *Vibrio anguillarum*, *Yersinia ruckeri* and *A. salmonicida*. In the vaccines against *V. anguillarum* and *Y. ruckeri* the protective antigens are considered to be LPS (Ward *et al.*, 1985; Ellis, 1988; Smith, 1988), and protection is serotype-specific. In contrast, in furunculosis the LPS does not seem to be important in generating a protective immune response. According to the work of Hirst and Ellis (1994) the antibody response directed against iron-regulated outer membrane proteins is correlated with protection, although a second antigen which enhances protection when incorporated into vaccines is "soluble lipopolysaccharide" (Bricknell *et al.*, 1997), a compound probably synonymous with CPS, given the similarities in properties (Garrote *et al.*, 1992; Garduno and Kay, 1995).

Although crude LPS for use in vaccines can be prepared quite simply from most organisms by heating the bacterial cells at 100°C (Campbell, 1964 In: Methods in immunology), thus denaturing protein antigens, other organisms may be more difficult to culture in quantities required for large-scale use in vaccines. An example of this is the intracellular bacterium *Piscirickettsia salmonis*, which must be grown *in vivo* or in tissue culture cells (Fryer *et al.*, 1990). Although a molecular biological approach is feasible for expression of protein antigens, e.g. outer membrane proteins, of such an organism, this could not be readily applied to polysaccharide antigens. Thus, the possibility that polysaccharide epitopes could be mimicked by peptides is worthy of investigation, and for this purpose the *A. salmonicida* antigens LPS and CPS were chosen as model systems. When this work began, it had been reported that phage display libraries had been used to identify peptides mimicking sugars or oligosaccharides such as α-D-methylmannoside (the ligand recognised by concanavalin A; Oldenburg *et al.*, 1992; Scott *et al.*, 1992), the Lewis^y blood group antigen (Hoess *et al.*, 1993), and the receptor for the cell adhesion molecule, E-selection (Martens *et al.*, 1995).

The interaction between the binding site of an immunoglobulin molecule (or paratope) and an epitope of an antigen arises from complementarity in shape, charge

distribution and the opportunity for hydrogen bonding to occur (Eisen 1990 In: Microbiology 4th ed.). It has been predicted by Barlow (1986) from consideration of the surface properties of proteins that most epitopes on proteins will be discontinuous, i.e. comprised of amino acids adjacent to each other in the tertiary structure but not in the primary amino acid sequence. The production of mimotopes, linear peptides which mimic the discontinuous epitope (Geysen *et al.*, 1986), and internal image monoclonal antibodies, capable of mimicking an epitope recognised by a monoclonal antibody (Poskitt *et al.*, 1991) suggests that a search for peptides which mimic polysaccharide epitopes may be worthwhile.

Two different strategies were adopted in this work, the first using a polyvalent antiserum to *A. salmonicida* CPS, in which it was decided to elute bound phage by displacing it with the native antigen, CPS. This procedure ought to displace only those phage binding to the anti-CPS binding sites, and in successive rounds select phage of higher affinity. The second approach was the conventional method of using a monoclonal antibody, in this case to *A. salmonicida* LPS, in which all bound ligands in the biopanning process would be identical.

4.1 Selection of phage binding to anti-CPS by biopanning from a 6-mer phage display peptide library

In the two biopanning experiments summarised in Table 2 (p.42), the percentage recovery of phage was low and did not increase during the three rounds of biopanning. However, the ability of phage to bind to anti-CPS antibody was screened to identify which might have an affinity for antibody.

It was initially considered that those phage which gave absorbance values above background level had a moderate to high affinity towards the anti CPS antibodies, and those with low, or background, absorbance had a low affinity. However, the low absorbances may have arisen because the concentration of certain amplified phage

was too low to produce sufficient binding sites for the anti-CPS antibodies. When the effect of concentration of phage on the ELISA absorbance was tested with 'high affinity' phage, Figure 17 showed that a dilution of 1/10 and 1/32 was sufficient to reduce the absorbance of the 'high affinity' phage from clones 1 and 2, respectively, to background level and equal to that of the low binding phage from clone 6. From these results it was concluded that the phage which yielded absorbances above background level were present in adequate concentration and displayed an affinity for the anti-CPS antibody. However, those phage giving an absorbance at or below background may either be those with a low affinity, or phage with a high affinity which had not been amplified to a sufficiently high concentration to be detected by the ELISA method. As previously noted, this would only require the reduction of amplification of phage by one tenth that of the high affinity clone 1.

The consistency of the ELISA method was demonstrated by the results shown in Figure 16, when comparing six repeat samples for each phage. These results were consistent within the experiment and were comparable with the initial ELISA results (Figure 15) for the clones tested. Further evidence that the amplification process led to widely differing yields of phage was obtained when ELISA was used with anti-M13 antiserum to detect the phage amplified after selection with MAb to LPS (Figure 24).

To determine whether there was indeed any affinity of the selected phage towards the antigen binding site of the antibody it was decided to carry out a competitive ELISA. The first point to be revealed from the results in Figure 18 was that phage 6, which was initially deemed a low affinity clone, is in fact a high affinity clone when assayed with the re-amplified phage 6 stock used for this experiment. The results of the competitive ELISA were inconclusive in that there was no clear dose-dependent inhibition by CPS of the binding between phage and antibody. Although the low concentrations of CPS inhibited binding of phage to antibody the high concentrations were not inhibitory. In all cases, concentrations of 1 μg/ml or 0.32 μg/ml CPS

inhibited the quantity of phage bound (Figure 16). It is possible that the ratios of CPS, antibody and phage favoured the formation of mixed complexes, comprised of phage and antibody interacting with CPS. Nevertheless, the high absorbances obtained with control phage in the absence of CPS provide further evidence that the phage have an affinity for the anti-CPS antibodies. The poor ELISA signal when CPS was bound to the plates as a positive control to detect anti-CPS binding was attributed to the well-recognised difficulty in obtaining good binding of polysaccharide antigens to microtitre plates under conditions normally used for binding protein antigens (Bantroch *et al.*, 1994). Preliminary experiments were done to verify the best conditions for binding CPS using various concentrations of CPS and various pH (Figure 19). For concentrations of 20-160 µg/ml CPS the best pH for binding was pH 9 with the amount of CPS bound being concentration dependent. However, further work on the ELISA methodology was discontinued as it was considered more profitable, in the limited time available, to determine the DNA and hence peptide sequences of the inserts. This involved the sequencing of a section of the phage DNA containing the eighteen-base-variable oligonucleotide.
For sequence determination, phage which appeared to have a high affinity for the anti-CPS antibody were chosen, along with a selection of 'low affinity' phage.

Of the seventeen phage for which the insert sequences were determined it was interesting to note that ten of these contained inserts with a GLY SER GLY motif in the first three amino acids. Equally important, the subsequent three amino acids of the 'GLY SER GLY' group of phage were of variable sequence. This suggests that these phage were selected for because of their 'GLY SER GLY' motif rather than, for example, if their genetic coding sequences had been identical and the phage had been present in a large copy number in the library. In the latter case the phage could have passed through the biopanning process purely because of their quantity rather than because of selection.

Partial similarities, such as those obtained, suggest that the serine and glycine residues in the GLY SER GLY format are being selected for. The serine residue contains an hydroxyl group which may be recognized by the anti-CPS antibody since each of the sugar units found in the CPS also contain hydroxyl groups (Appendix 3). If this is the case, the occurrence of other hydroxyl containing amino acids, threonine and tyrosine, might also be expected. Threonine does indeed appear in sequence 1 (Figure 20) as part of the motif 'GLY THR GLY SER' which is very similar to the insert sequence of phage 4 , 'GLY SER GLY SER'. These motifs may mimic CPS to a greater extent than those containing only one hydroxyl side chain since alternate hydroxyl-containing amino acids may mimic the hydroxyl groups and their spacing on the various sugars in CPS. Phage 18 insert, which contains the motif 'PRO TYR SER PRO' is interesting in that not only does it contain the hydroxyl carrying amino acids TYR and SER but these are flanked by the 'kink forming' amino acid proline which may cause the TYR SER residues to protrude from the surface of the PIII protein on which it is displayed, perhaps making these hydroxyl-carrying amino acids more accessible to the anti-CPS antibody.

As previously noted, all clones sequenced from this round of biopanning contained the amino acid serine, including the four clones assessed by ELISA as being of low binding affinity. As discussed previously, these phage may have been erroneously labelled as having low binding affinity because they were not amplified to sufficiently high concentration for the ELISA. This is especially relevant for phage 14 and 20 which contain the distinctive motif 'GLY SER GLY'. Since these clones are identical, it is possible that the insert may have had greater effect on the infectivity of the phage than other insert sequences, i.e. the insert may have disrupted the function of the PIII protein and thus reduced infectivity, which would in turn reduce the yield of the phage on amplification.

A further unexpected finding was that one of the sequences contained only two amino acids (Figure 22); however, the sequence determined, 'GLY SER', was very similar to the common motif 'GLY SER GLY' noted above. The 'GLY SER' motif, although apparently shorter than the 'GLY SER GLY' motif discussed above, is actually expressed as 'GLY SER GLY' because glycine is the first amino acid expressed in the non-variable section of the PIII protein. The unusual occurrence of a two amino acid insert is discussed later but its selection here was further confirmation of the power of the biopanning process.

After re-amplification of the second round eluate from the CPS biopanning and continuing to a third round, the phage selected from this round were again assayed by ELISA. Few clones gave significant positive results (Figure 23) and for this reason a semi-quantitative assay for phage concentration was developed using an anti-phage M13 antibody ELISA (Figure 24). With this technique it was possible to determine whether particular phage had been amplified to a detectable concentration, and showed that approximately one third of the clones required further amplification.

Analysis of the sequences, yielded the surprising result that the previously determined motif 'GLY SER GLY' was not found. The reason for this is unclear. However, variations of the motif, with the amino acids replaced by those with similar functional groups, did occur. This can be seen in that many of the inserts contained an hydroxyl- carrying amino acid, flanked by various members of the aliphatic amino acid group. Examples are phage 59 and 25 with the motifs 'LEU SER LEU' and 'GLY TYR LEU', respectively.

Further examination shows that of the 17 sequences which could be read, 13 contained hydroxyl carrying amino acids; of these, 7 contain more than one hydroxyl-carrying amino acid. Clone 54 is of particular interest since it contains 3 hydroxyl-carrying amino acids, viz. 'PHE TYR SER ALA SER ILE', which again shows a serine flanked by aliphatic amino acids (ALA and ILE). Again, it may be that the

hydroxyl groups which are carried by the serine and tyrosine amino acids are recognized by the anti-CPS antibodies.

As found in the previous experiment a non 6-mer phage was detected, however, this phage displayed a 5 amino acid insert rather than a two amino acid insert. It may be that these unusual phage occur with a high frequency in the library and hence appear at an equivalent frequency after biopanning. However, since both the 2-mer and 5-mer contained the amino acid serine, it is suggested that they are selected for to a high frequency via the biopanning process. The occurrence of non 6-mer phage and wild type phage (clone 78) are discussed later.

Work by Hoess *et al.* (1993) deduced that the consensus amino acid sequence APWLYGPA was selected by a monoclonal antibody (B3) raised against a carbohydrate antigen. It is interesting to note the similarity between the LYG (LYS TYR GLY) moiety in this sequence with the 'GLY SER GLY' moiety, and similar combinations, of hydroxyl-carrying amino acids flanked by two aliphatic amino acids obtained in the previously discussed experiments with anti-CPS antibody. Since the anti-carbohydrate antibody recognises the sugar units of the carbohydrate it is not surprising that similarities occur between the phage obtained using anti-CPS and those using other anti-carbohydrate antibodies. It would, however, be interesting to test the extent of cross reactivity between these antibodies against both sets of phage.

4.2 Biopanning a 6-mer phage library with a monoclonal antibody to LPS

After the three rounds of biopanning with the 6-mer phage library and monoclonal anti-LPS antibody, selected phage were assayed with ELISA, but there was no indication (Figure 28) that any selection has occurred. However, it should be noted that the LPS control concentrations of 10 & 33 µg/ml did not give absorbance values as high as in previous experiments, indicating a possible problem with at least one of the many ELISA steps or components. Despite carrying out various tests to improve

the ELISA system, it was deemed more productive to carry out sequence determination for inserts of the selected phage than to pursue the problem further.

The most obvious findings from the phage sequences were the occurrence of groups of up to eleven phage with identical sequences and that, secondly, of the 70 readable sequences, 52 contained hydroxyl side-chain carrying amino-acids. The most common sequence was 'ASN LEU MET ARG LEU TYR' which not only appeared eleven times but was also isolated from each of the four separate tubes (A, B, C and D) during biopanning.

When compared with the finding for CPS biopanning, the phage selected during biopanning with anti-LPS had similar motifs, i.e. with hydroxyl-carrying amino acids flanked by aliphatic amino acids, typified by the sequence 'ALA SER ILE' in phage 53 and 'VAL TYR ALA' in clone 4. Additionally, sequences such as 'LEU MET ARG' found in phage 80 are found in reverse in phage 82; similarly, the sequence "TRP ARG HIS' found in phage 47 is found in reverse in phage 68. Phage 47, and those phage isolated with the same sequence, are of interest since they appear to have been selected yet contain no hydroxyl-carrying amino acids. This same observation can be applied to phage 68 and 58 which have the same sequence yet have different tubes of origin. As mentioned previously, there is a common motif between these phage, the sequence 'TRP ARG HIS'. Also, two amino acids distant from the TRP in either direction, the amino acid ARG occurs. This suggests that these amino acids may also be recognized by the anti-LPS antibody. Further tests with ELISA require to be carried out to verify whether these phage have an affinity for the anti-LPS antibody. This experiment, as with the CPS biopanning experiment, yielded some unexpected sequences. Phage 57 carried the wild type Gene III sequence and phage 18 contained a five amino acid insert.

4.3 Identical phage sequences selected during anti-CPS biopanning and anti-LPS biopanning.

The occurrence of identical phage sequences obtained from anti-CPS biopanning with those obtained with anti-LPS biopanning, as noted above, could be due either to an excess representation of these phage in the 6-mer stock library, causing them to pass through the biopanning system via quantity rather than via selection or, it could be due to recognition of similar epitopes by the anti-CPS and anti-LPS antibodies. The composition of LPS and CPS is very similar (Appendix 3.), but further experiments are required to test the degree of cross-reactivity between the sequences obtained and the anti-CPS and anti-LPS antibodies. The possibility that a common sequence on the rabbit and mouse immunoglobulin is recognised, cannot yet be discounted.

4.4 Biopanning with a 15-mer phage library and anti-LPS antibody

As noted earlier (results section 3.8.3), the phage selected from the 15-mer library gave ELISA results (Figure 28) similar to the control background level but, nevertheless, it was decided to sequence a small number of the phage selected. The inability to detect 15-mer insert sequences was surprising, but as the two insert sequences were identical to those of phage 22 and 32 from the anti-LPS 6-mer biopanning experiments, the possibility of cross-contamination cannot be ruled out. However, the frequency with which wild-type sequences, i.e. no insert, were formed, was much higher than with the 6-mer library (3/5 compared with 1/50 for the 6-mer library) which indicates that the origin of the 15-mer library is most unlikely to be the 6-mer library used in the study.

4.5 Occurrence of wild type phage and non-6-mer sequences

The occurrence of the wild type Gene III sequence, although unexpected, was not unusual as it is well recognised that this can occur in construction of libraries. The electron microscopy discounted the possibility that the host *E. coli* K91 Kan carried the wild type fd phage (containing no antibiotic resistance) since no phage were detected in non-infected cells whereas they were readily seen in infected cells.

As stated by Bonnycastle *et al.* (1996), the quality of a phage library can be assessed in terms of several parameters. These include the number of clones in the library, the level of peptide expression on the virion and the errors in the DNA sequences encoding the peptide inserts. In the 11 phage libraries characterised by Bonnycastle *et al.* (1996) the occurrence of wild type sequences varied from 0% to as much as 19%, and the occurrence of unexpected or 'deviant' insert sizes ranged from 0% to 17.6%.

Thus the finding of a low frequency of wild-type and deviant sequences in the 6-mer library (2% and 3% respectively), suggest that it was satisfactory in this respect. For comparison, the 6-mer library of Bonnycastle *et al.* (1996), contained 7% wild-type sequences and 0% deviant sequences.

4.6 Selection of phage sequences which do not mimic an antigenic determinant of LPS or CPS.

The assumption implicit in the discussion so far is that the sequences selected have been related to the structure of LPS or CPS since they were selected by the anti-LPS or anti-CPS antibodies. However, it is possible that the phage may have been selected because of their reactivity against streptavidin, biotin or even the plastic of the tubes. Selection of phage against streptavidin or plastic is unlikely since biotin or plastic surfaces, if not bound by antibody, should have been blocked before the phage was added. However, the biotin molecule may be exposed since each antibody molecule has, on average, several covalently-bound biotin molecules, and not all would be used to bind to the streptavidin. Robert *et al.* (1993) determined a consensus sequence of 'X X TYR TYR LEU HIS' for phage isolated using anti-biotin antibody. This sequence is reminiscent of many of those obtained using selection with anti-LPS antibodies since the motif of 'LEU TYR' occurs in many of these sequences. However, the greatest similarity to this 4 amino- acid consensus is that of

phage 87 from anti-LPS biopanning which has the 3 amino acid sequence of TYR LEU HIS. The sequence for phage 87 suggests that this phage may perhaps have greater affinity towards biotin than to anti-CPS antibody. Closer inspection of the sequences obtained by other workers (Robert *et al.*, 1993) for anti-biotin or streptavidin biopanning shows that none of the sequences obtained contained the amino acids serine or threonine. This suggests that the phage selected here which contain these amino acids are less likely to have been selected because of an affinity to biotin or streptavidin.

4.7 Implications of more recent research

During the writing of this thesis other work has been published which is relevant to this project. Bonnycastle et *al* . (1996) showed that they also experienced a certain occurrence of wild type phage and expressed concerns about the quality of the synthetic oligonucleotide used, and mention "toxicity" of certain peptide inserts. Interestingly, as deduced in this project, they note that certain phage varied in their amplification in *E. coli* by tenfold or greater. Bonnycastle attempted to obtain mimotopes to polysaccharide antigens using monoclonal antibodies D1, D2 and D3, respectively specific for *Salmonella paratyphi* (branched trisaccharide of the O-antigen of the LPS), *Shigella flexneri* (tetrasaccharide of the O-antigen of the LPS) and *Streptococcus pyogenes* (branched trisaccharide of the cell-wall polysaccharide). The consensus sequences deduced from phage selected were: for D1, the sequences YPM and TYVLTC ; for D2, CXNM, and for D3 CXLY and CXXLY. Cross reactivity occurred between these peptides and the anti-carbohydrate antibodies. It should be noted that there are similarities between the above peptides and those obtained within this study, especially the occurrence of the LY motif obtained using anti-CPS and anti-LPS antibodies.

More important advances were made by Phalipon *et al.* (1997) who obtained two immunogenic peptide sequences which were shown to mimic the O-antigen of *Shigella flexneri.* The phage selected using monoclonal antibody to the LPS

contained several sequences including YKPLGALTH and KVPPWARTA. Similarities between these peptides and those shown within this thesis can be observed with respect to the hydroxyl-carrying amino acids. The most important finding of Phalipon *et al.* was that phage displaying the above peptides could induce an immune response in mice, the antibodies cross-reacting with the O-antigen of *Shigella flexneri*. This is the first reported example of immunogenic mimicry of carbohydrate by phage-displayed peptides. Although the above peptides which mimic polysaccharides appear to be specific, Bonnycastle *et al.* (1996) suggest that in their work, due to similarities in saccharide structure, the phage isolated are cross-reactive with other anti-saccharide antibodies. In contrast to this, Harris *et al.* (1997) showed that peptides selected by different anti-saccharide antibodies remained specific to the antibody which selected them. It should be noted that Bonnycastle *et al.* (1996) have not reported any results of immunisation with the phage-displayed peptides.

A separate approach to produce an effective vaccine using LPS may lie in understanding and utilising internal image anti-idiotype vaccines. As described in the reviews by Poskitt *et al.* (1991) and Dalgliesh and Kennedy (1988), antibodies raised against a monoclonal antibody may bear an internal image of the antigen to which the monoclonal was raised, in accordance with Jerne's Network Theory (Jerne, 1974). This antibody is known as the Ab2β anti-idiotype and may be used to induce an immune response. This system is thought to play a role in the transfer of immunity from mother to foetus. This is important since neonates up to 2 years have poor immunogenicity against polysaccharide antigens and would therefore benefit from passive immunisation to polysaccharides by polysaccharide-mimicking antibodies. Mimicry of polysaccharide antigens by anti-idiotypic antibodies has been studied by Field and Morrison (1994), who demonstrated that an anti-idiotypic antibody which mimicked the inner core of *Salmonella minnesota* LPS, protected mice from a normally lethal challenge with LPS. Many other examples exist in the literature.

This research would suggest that production of anti-idiotypic antibodies against polysaccharide-mimicking peptides selected from bacteriophage-displayed peptide libraries may lead to an effective means of inducing an immune response in salmonid fish to the pathogen *Aeromonas salmonicida.*

4.8 Further work arising from this thesis

Only a very limited time was available to complete the work described in this thesis and it is recognised that a more thorough characterisation of the affinity of binding of phage to the antibodies is required.

Secondly, the sequences which either showed a good response with ELISA or have interesting amino acid sequences, should be tested for their ability to induce an immune response in fish and in mice. Serum samples could subsequently be collected and tested for anti-CPS and anti-LPS antibody production.

One major difference between this study and those of Bonnycastle *et al.* (1996) and Phalipon *et al.* (1997) is that these workers concentrated on phage libraries with peptides displayed in the virion major coat protein (pVIII). This has the significant advantage of displaying several hundred copies of the peptide insert rather than the two or three copies per virion displayed in pIII libraries. However, it is considered that sufficient progress has been achieved so far in this study to warrant further experiments, perhaps with pVIII libraries.

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APPENDICES

Appendix 1

Reagents used in biopanning

Elution buffer

0.1 N HCl. pH adjusted to 2.2 with glycine
1 mg/ml BSA
0.1 mg/ml phenol red (optional)
The glycine/HCl buffer is made and adjusted as a 4x stock, filter sterilised, and stored at room temperature.

Phage storage buffer (if needed)

0.01 M Tris base
0.01 $MgCl_2$ $6H_2O$

Potassium phosphate buffer

0.17 M KH_2PO_4 (anhydrous)
0.72 M K_2HPO_4 (anhydrous)

Tris buffered saline

50mM Tris/HCl pH7.5
150mM NaCl

TBS/Tween solution

200 vol TBS
1 vol Tween 20 (final conc. 0.5% v/v)

Bacteria, culture media

E. coli K91kan

A "mini-kan hopper" was inserted into the *lacz* gene of K91 *Escherichia coli* with the aid of the catalyst vector λNK1105 consequently introducing

kanamycin resistance to this organism (Way *et al.*, 1984). The strain was obtained from Dr. G. Smith (ref: SE94)

Luria agar

15 g/l agar (technical no.3)
10 g/l bacto-tryptone
10 g/l NaCl
5 g/l yeast extract

Luria broth

10 g/l Bacto-tryptone
10 g/l NaCl
5 g/l yeast extract

Terrific broth

To 900 ml water add
12 g bacto-tryptone
24 g yeast extract
4 ml (5.04 g) glycerol
Autoclave 90 ml portions and to each add 10 ml autoclaved potassium phosphate buffer

Kanamycin

100 mg/ml stock

Tetracycline (hydrochloride)

40 mg/ml and 10 mg/ml stock solution (dilute was H_2O rather than Ethanol)

C) Additional reagents for ELISA

Blocking solution

0.1 M $NaHCO_3$
5 mg/ml dialysed BSA
0.1 µg/ml streptavidin
0.02% NaN_3 (optional) (store at 4 °C)

Appendix - 2

Sequencing procedure

The Sequenase^{TM} version 2.0 DNA sequencing kit. (Product No. us 7077) was used.

Step 1

For sequencing of single stranded DNA the annealing mixture consists of 7 µl DNA (prepared from bacteriophage as described above), 2 µl reaction buffer and 1 µl primer (0.5 pM). The mixture was heated at 65°C for 2 min, then cooled to 35°C over a 15-30 min period before being chilled on ice until it was used in step 5.

Step 2

Eppendorf tubes labelled G, A, T and C each received 2.5 µl of termination mixture (dGTP).

Step 3

The labelling mix was diluted 5 fold (2 µl plus 8 µl H_2O).

Step 4

The Eppendorf tubes labelled G, A, T & C were then placed in a 37°C bath.

Step 5

The labelling reaction was prepared as follows;

Ice chilled annealed DNA mixture from step 1	10 µl
Dithiothreitol, 0.1M	1 µl
Diluted labelling mix	2 µl
Redivue^{TM}[^{35}S] dATP	0.5 µl
Sequenase polymerase (diluted 8-fold)	8 µl

The components were mixed and incubated at room temperature for 2-5 min.

Step 6

Termination reactions.

Aliquots of 3.5 µl of labelling reaction were transferred to each termination tube (G, A, T, C). The tubes were then mixed and allowed to incubate at 37°C for 5 min.

Step 7

The reactions were stopped after 5 min by the addition of 4 µl of stopping solution.

Step 8

The samples were heated to 75°C for 2 min prior to running the sequencing gel.

Preparation and running of polyacrylamide sequencing gels

The major components are "Easy gel" the ready prepared reagent Acrylamide/Bis-acrylamide 6% sequencing gel (ratio 19-1 7M Urea 1 x TBE) supplied by Scotlab (Product No. SL-9238), Temed (N,N,N,N-Tetra-methylethylenediamine) from Sigma (Product No-T8133) and Ammonium persulphate in the following amounts

Easy gel 70 ml

Temed 70 µl

Ammonium persulphate 140 µl (0.125 g/500 µl H_2O) prepared directly before use)

The Gel plates were 42 x 33 cm and were mounted in a Model S2 sequencing apparatus from Life technologies Ltd with power supplied by a Gibco BRL electrophoresis power supply (model No. 2500).

The mixture was poured immediately into previously taped glass plates, the small plate being pre-treated with 'gel slick™' (A non toxic glass plate coating from AT Biochem, catalogue No. 219). The comb was inserted approximately 1 cm into the gel with the points facing outwards. The gels set after 1.5 h at which time the comb was reversed and inserted so that the points of the comb pierced the gel by approximately 1 to 2 mm. The gel was then loaded into the apparatus and the top and bottom reservoirs filled with TBE buffer. A syringe with a needle was used to wash TBE buffer over the wells to displace any residual urea that would interfere with loading the samples.

Aliquots of 3.5 µl of each sample were loaded in the order T, C , G and A. The gel was then run for 2.5 -3 h at 300 mA / 80 W or 280 mA / 70 W. To facilitate reading the sequence of the gel it was found best to allow the fragments containing the sequence of

interest to run to the end of the gel. A good indication of when this was achieved was when the first of the two dyes in the stop solution had just run of the end of the gel, which normally took 2.5 -3 h.

The smaller plate was removed and the gel was transferred from the larger plate by placing a sheet of filter paper on top of the gel and lifting it away, leaving the gel stuck to the paper in its original orientation. The gel was vacuum dried at 60°C for at least 3 h. To determine the appropriate exposure time required for the film, the radioactivity of the dried gel was tested using a Geiger counter. In general, when counts (Using a mini-monitor g.m.meter type 5.1.0 from Mini Instruments) of 50/sec and above were obtained, the film was exposed to the gel for 12 - 24 h; however, when lower counts were obtained the gels were placed against film for as long as 14 days to produce bands which were dark enough to read. Following development the sequence of the gel was determined. For the 6-mer library, reading of an 18 base pair (bp) sequence was required, and for the 15-mer library a 45 bp sequence encoded the peptide sequence. These sequences were located by looking for the distinctive pre-sequence and post-sequence patterns, TGCCCCGA and CCCCGGCGACCCCGG respectively. The 'insert' sequence was easily identified by locating the three groups of four cytosine residues, from which the insert sequence was read directly from the film and a complementary strand constructed. The three bases immediately upstream from the insert G-C-T formed an alanine codon; this amino acid was be used to translate the reconstructed sense-strand through the insert and into the region coding for pIII.

Appendix 3 - COMPOSITION OF LIPOPOLYSACCHARIDE AND CAPSULAR POLYSACCHARIDE

LPS	Molar ratios
Glucose,	1.35
rhamnose,	1
N-acetylmannosamine,	1
(Partially acetylated, estimated from Fig. 1)	

CPS	
Glucose,	5
mannose,	3
rhamnose,	0.75
N-acetylmannosamine	2
mannuronic acid	1

from Shaw *et al.*, (1983) and Garrote *et al.*, (1992)

Appendix 4 - Bacteriophage fd sequence (from EMBL data base) accession no V00602

Genome of the bacteriophage fd (Inoviridae). 6408 bp of circular DNA [Beck,E., *et al* 1993, Nucleic Acids Res. 5 (12), 4495-4503 (1978)]

GENE TRNSLATION

GENE II="MIDMLVLRLPFIDSLVCSRLSGNDLIAFVDLSKIATLSGMNLSARTVE
(226 - 1458)
YHIDGDLTVSGLSHPFESLPTHYSGIAFKIYEGSKNFYPCVEIKASPAKVLQGHNVFG

TTDLALCSEALLLNFANSLPCLYDLLDVNATTISRIDATFSARAPNENIAKQVIDHLR

NVSNGQTKSTRSQNWESTVTWNETSRHRTLVAYLKHVELQHQIQQLSSKPSAKMTSYQ

KEQLKVLSNPDLLEFASGLVRFEARIETRYLKSFGLPLNLFDAIRFASDYNRQGKDLI

FDLWSFSFSELFKAFEGDSMNIYDDSAVLDAIQSKHFTITPSGKTSFAKASRYFGFYR

RLVNEGYDSVALTMPRNSFWRYVSALVECGIPKSQLMNLSTCNNVVPLVRFINVDFSS

QRPDWYNEPVLKIA"

GENE X="MNIYDDSAVLDAIQSKHFTITPSGKTSFAKASRYFGFYRRLVNEGYDSV
(1123 TO 1458)
ALTMPRNSFWRYVSALVECGIPKSQLMNLSTCNNVVPLVRFINVDFSSQRPDW

YNEPVLKIA"

GENE V="MIKVEIKPSQAQFTTRSGVSRQGKPYSLNEQLCYVDLGNEYPVLVKITL
(1470 TO 1733)
DRGQPAYAPGLYTVHLSSFKVGQFGSLMIDRLRLVPAK"

GENE VII="MEQVADFDTIYQAMIQISVVLCFALGIIAGGQR"
(1735 TO 1836)

GENE IX="MSVLVYSFASFVLGWCLRSGITYFTRLMETSS"
(1833 TO 1931)

GENE VIII="MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAFDSLQASATEYIG
(1928 TO 2149)
YAWAMVVVIVGATIGIKLFKKFTSKAS"

GENE III="MKKLLFAIPLVVPFYSHSAETVESCLAKPHTENSFTNVWKDDKTLDR
(2206 TO 3480)
YANYEGCLWNATGVVVCTGDETQCYGTWVPIGLAIPENEGGGSEGGGSEGGGSEGGGT

KPPEYGDTPIPGYTYINPLDGTYPPGTEQNPANPNPSLEESQPLNTFMFQNNRFRNRQ

GALTVYTGTVTQGTDPVKTYYQYTPVSSKAMYDAYWNGKFRDCAFHSGFNEDPFVCEY

QGQSSDLPQPPVNAGGGSGGGSGGGSEGGGSEGGGSEGGGSEGGGSGGGSGSGDFDYE

KMANANKGAMTENADENALQSDAKGKLDSVATDYGAAIDGFIGDVSGLANGNGATGDF

AGSNSQMAQVGDGDNSPLMNNFRQYLPSLPQSVECRPYVFGAGKPYEFSIDCDKINLF

RGVFAFLLYVATFMYVFSTFANILRNKES"

GENE VI="MPVLLGIPLLLRFLGFLLVTLFCYLLTFLKKGFGKIAIAISLFLALII
(3483 TO 3821)
GLNSILVGYLSDISAQLPSDFVQGVQLILPSNALPCFYVILSVKAAIFIFDVRQKIVS

YLDWDK"

GENE I="MAVYFVTGKLGSGKTLVSVGKIQDKIVAGCKIATNLDLRLQNLPQVGRF
(3824 TO 4870)
AKTPRVLRIPDKPSISDLLAIGRGNDSYDENKNGLLVLDECGTWFNTRSWNDKERQPI

IDWFLHARKLGWDIIFLVQDLSIVDKQARSALAEHVVYCRRLDRITLPFVGTLYSLVT

GSKMPLPKLHVGVVKYGDSQLSPTVERWLYTGKNLYNAYDTKQAFSSNYDSGVYSYLT

PYLSHGRYFKPLNLGQKMKLTKIYLKKFSRVLCLAIGFASAFTYSYITQPKPFVKKVV

SQTYDFDKFTIDSSQRLNLSYRYVFKDSKGKLINSDDLQKQGYSITYIDLCTVSIKKG

NSNEIVKCN"

GENE IV="MKLLNVINFVFLMFVSSSSFAQVIEMNNSPLRDFVIWYSKQTGESVIV
(4848 TO 6128)
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NUCLEOTIDE SEQUENCE

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601 CCTTGCTTGT ACGATTTATT GGATGTTAAC GCTACTACCA TTAGTAGAAT

651 TGATGCCACC TTTTCAGCTC GCGCCCCAAA TGAAAATATA GCTAAACAGG

701 TTATTGACCA TTTGCGAAAT GTATCTAATG GTCAAACTAA ATCTACTCGT

751 TCGCAGAATT GGGAATCAAC TGTTACATGG AATGAAACTT CCAGACACCG

801 TACTTTAGTT GCATATTTAA AACATGTTGA ACTACAGCAC CAGATTCAGC

851 AATTAAGCTC TAAGCCATCC GCAAAAATGA CCTCTTATCA AAAGGAGCAA

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